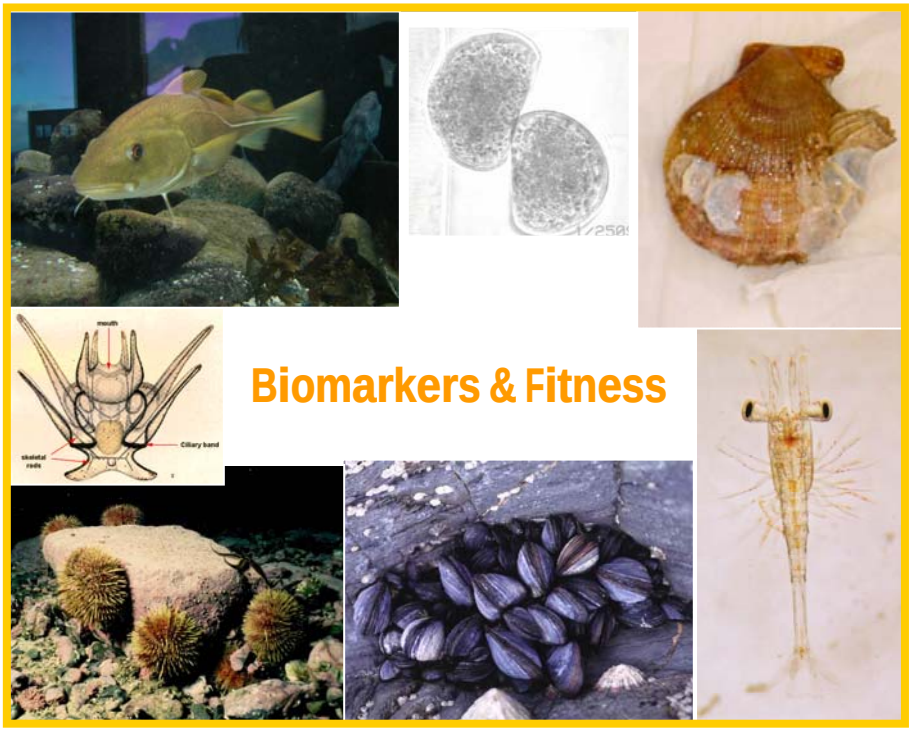
BioSea JIP Program

BioSea JIP summary report of results from laboratory experiments



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1 Introduction

This report provides an overview of the results obtained from biomarker analyses carried out on Molluscs, Crustaceans, Echinoderms and Fish, together with measurements of fitness parameters examined in shrimps and mussels, all performed within the BioSea JIP programme.

In the BioSea JIP experiments that used invertebrates, fitness parameters in early life stages were studied following long term exposure of parent animals (mussels, sea urchins) or long term exposure of embryos (shrimps). In addition, a number of biomarker responses were analysed. The selection of biomarkers was based on results from previous oil exposure experiments at Akvamiljø, together with recommendations taken from the literature. To be able to study possible effects on fitness parameters, invertebrates were exposed to oil for several months. Samples for biomarker analyses were taken following one month's exposure, a time period typically employed in biomonitoring studies using caged organisms. Samples for biomarker analyses were also taken at the end of the exposure (3-7 months) to compare the biomarker responses in adult animals to the potential effects on early life stages.

Only biomarker responses were studied in the two long term cod exposure experiments. A two week recovery period and more frequent sampling for biomarker analyses were included in the fish exposures. In connection to the BioSea JIP experiments additionally exposed animals and analyses funded by the Norwegian Research Council through the PROOF program are included in the data summarized in this report. (NF project: 153882/S40: Validation of methods and data for Environmental Risk Assessment off-shore).

Results from statistical comparisons of biomarker responses and effects between oil exposed and control animals will be presented in tables and discussed. Based on these findings recommendations for the applicability of each biomarker in biomonitoring studies will be made. Additional statistical analysis has not been undertaken for this report. Multivariate treatment of the data and/or calculation of Integrated Biomarker Response (IBR) (presented by T. Baussant at the last BioSea workshop in Stavanger) present a possibility for further treatment of data in the planned publications. Details about the type of statistical tests used and discussion of each data set is provided within

the written reports for each individual project (Baussant, 2004, Larsen, 2004, Skadsheim, 2004 and Bechmann, 2004).

1.1 Experimental design

Two series of experiments have been performed in which fish and invertebrates were exposed to oil; one series with Statfjord B oil at 7-10ºC, and one series with Goliat oil at 3-5ºC.

Blue mussel (*Mytilus edulis*), Northern Shrimp (*Pandalus borealis*) and green sea urchins *(Strongylocentrotus droebachiensis)* were exposed to three concentrations of dispersed Statfjord B oil under North Sea conditions (temperature 7-10°C) in 2002. The concentrations of oil in the tanks were regularly monitored by fluorescence measurements in order to assess the total hydrocarbon content (THC). Based on fluorescence, THC in the seawater of the low, medium and high exposure tanks were in the ranges of 3-4 µg/l , 15-29 µg/l and 63-90 µg/l THC (oil) respectively. The three invertebrate species were exposed in separate tanks in a continuous flow exposure system (CFS). Mussels and sea urchins were exposed for 7 months, shrimps for 5 months (3 months for the highest exposure due to mortality).

Arctic scallops (*Chlamys islandica*) and Northern Shrimp (*Pandalus borealis*) were exposed for 1 month to Goliat oil under Arctic conditions (5ºC) in a separate experiment in 2003. Based on fluorescence, THC in the seawater was 2, 14 and 64 µg/L THC for the scallops, and 5, 23 and 102 µg/L for the shrimps.

Juvenile North Sea cod (*Gadus morhua* L.) were exposed to North Sea crude oil (Statfjord B) at 10ºC in 2002 and Juvenile Barents Sea cod (separate stock of cod) were exposed to Barents Sea crude oil (Goliat) at 3-4 ºC in 2003. In both experiments the fish were exposed to the nominal concentrations 0.06, 0.25 and 1 mg/L oil in addition to 1 mg/L oil spiked with 0.2 mg/L alkylated phenold and 0.2 mg/L of a mixture of PAHs for one month followed by a two week depuration period.

This is the first time that concentrations as low as these have been used at Akvamiljø and the exposure period was in excess of anything previously attempted.

Utvik and Gärtner (2004) have used DREAM to estimate the concentration of PAHs at different distances from the Statfjord B field. The concentration of sum PAHs

(including naphthalenes) in the discharged produced water from Statfjord B was 2 mg/L (data from 2001, volume of produced water: 39 220 m^3/day). The calculated concentration of sum PAHs (including naphthalenes) in the sea 500 m from Statfjord B was approximately 0.15 µg/L based on dispersion modelling.

The concentrations used for these exposures were in the same range as those measured ~500 m from the Statfjord field, as predicted by the DREAM model (Utvik & Gärtner, 2004).

1.2 Biomarkers as early warning signals for reduced fitness

Depledge defined biomarkers as: ”Biochemical, cellular, physiological or behavioural variations that can be measured in tissue or body fluid samples or at the level of whole organisms that provide evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiation)” (Depledge, 1994).

Biomarker responses in exposed animals indicate that they have been exposed to contaminants, that they are trying to compensate for the exposure by inducing various defence mechanisms (detoxification), that they are trying to repair damage, or that damage has occurred. If the damage cannot be repaired and the animals suffer subsequent problems related to the exposure, any biomarker signal measured at a preliminary stage of biological disturbance can be considered to give an early warning of these later detrimental effects and as such provide a perfect tool for biomonitoring discharges from the (oil) industry. Biomarkers of exposure, as well as those of effect described above, have a key role to play in biomonitoring procedures. In their simplest form these biomarkers provide an indication of bioavailable contaminants in the surrounding water and provide information on possible bioaccumulation effects that may have consequences for various constituents of ecosystems. The energetic cost of detoxification processes may leave animals with reduced energy for catching prey, avoiding predators and reproduction, resulting in reduced population growth rate. This is a possible *indirect* effect of exposure to toxic compounds at the population level. An example of a *direct* effect is provided by the scenario whereby genotoxic compounds cause genetic damage to eggs and sperm leading to reduced fertilisation success, an increased number of deformed larvae, reduced survival, culminating in a reduced population growth rate. In these cases the biomarker signal may not give an early enough warning, but even a late warning is better than none at all. For monitoring

effects of discharges to water column organisms the only alternative today is chemical analysis of water samples and organisms. Sensitive chemical analysis are available for many compounds, but it is necessary to know which of the compounds are likely to cause effects, and whether there are additive, synergistic or antagonistic reactions present in the mix of discharged chemicals. A suite of biomarkers can give more valuable information about the potential effects of a discharge at a lower cost than a full range of chemical analysis of water and/or organisms if the objective is to decide whether there are compounds in the discharge causing harmful effects.

Benthic biodiversity studies are used to study effects on animals living in the sediments around oil platforms. This type of study gives a rather late warning, telling us when a statistically significant reduction of the biodiversity has occurred, that some species have disappeared or that others have increased in number. The biomarker approach has the potential to give a warning of biological impact before contaminants in the discharge affect the biodiversity of water column organisms.

The purpose of studying fitness parameters in parallel with biomarker responses was to investigate whether biomarkers were able to predict subsequent changes in fitness measurements. An objective was to find a set of biomarkers that produced signals at lower exposure concentrations of oil than the concentration causing reduced fitness. If a short exposure time induces a biomarker signal, while a much longer exposure time is needed to cause reduced fitness, the biomarker can also be considered to give an early warning.

1.3 Statistical and biological significance

Biomarker responses may vary between tissues, individuals and species, and the biomarker signal does not always increase (or decrease) with increasing exposure concentration or exposure time. There will be individual differences in sensitivity to toxic compounds, and in the efficiency of the repair processes. Hence the difference in the biomarker signal between control and exposed animals (even in laboratory experiments) will not always be statistically significant even when animals have been exposed and detoxification initiated. If the chosen biomarkers are not sensitive enough or if the method is not optimized for the species and tissue used (e.g. enough replicate samples) there is a risk of false negatives, indicating no effect when in fact one is present (a Type II error). Lam & Gray (2003) discuss the use of biomarkers in

environmental monitoring programmes, and they emphasize that Type II errors must be kept to an absolute minimum. In environmental terms making Type II errors is more serious than making Type I errors (false positives: indication of effect indicated by statistical analysis when in fact one is not present). To reduce false negatives we discuss trends in the data, to indicate where we may miss the early warning signal.

Statistical significance must not be confused with biological significance. If it is possible to take many replicate samples from each group of animals a very high significance level (low p value) can be obtained even when the differences between groups are small. One example is the Comet assay where we measure DNA strand breaks in several hundred cells in total (50 cells x 9 animals) from each treatment. A very small change in the level of DNA strand breaks can be statistically significant at the $p < 0.0001$ level. For obvious logistic/economic reasons it is not possible to have the same number of replicates from all types of biomarkers and especially not for fitness parameters. The number of larvae produced or the percentage of normal larvae may be 50 % reduced, but with 3-5 replicate groups of animals the difference may not be statistically significant at the $p<0.05$ level, which is the usual requirement for stating that a statistically significant effect has taken place. This could, however, be considered a significant biological effect.

It should be mentioned that there are inherent information in the measurements that are not utilized in the commonly used statistical tests, e.g. increase in variation and skewness. However, it would be beyond the level of detail aimed for in this compilation to bring in here.

1.4 Dose - response relationships for biomarkers

Mechanistic studies of dose-response relationships for biomarkers are usually carried out in the laboratory, either *in vitro* (exposed cells) or *in vivo* using very high concentrations of model compounds such as benzo(a)pyrene (injections are frequently used) and very short exposure time. It is difficult to extrapolate from this type of laboratory study to field conditions. The relatively low exposure concentrations of oil in the BioSea experiments were chosen to make it easier to extrapolate to field situations. The long exposure time in the invertebrate studies in BioSea were optimal for the early life stage test, but less optimal for detecting possible transient responses in enzymes like GST and Catalase.

Enzymes involved in compensating for exposure to toxic compounds (detoxification and repair) can be induced or inhibited depending on the concentration and type of contaminant and the exposure time. Metabolism of the contaminant may start a few hours following onset of the exposure and if extremely efficient it may be that enzyme activity cannot be detected very shortly after exposure ceases (non-linear dose-response curves, see figure 1). Increased activity of enzymes involved in detoxification may be sensitive early warning biomarkers, but because the dose-response relationships tend to be non-linear, such biomarkers are challenging when used for biomonitoring.

Figure 1 (bottom panel) shows a theoretical non-linear dose-response relationship. At relatively low concentrations the biomarker signal increases with increasing exposure concentration before a plateau level is reached, and finally the signal is reduced to below the control level at higher concentrations. Non-linear dose-response relationships may be relevant especially for enzymatic biomarkers (e.g. EROD, Catalase, GST) and possibly for biomarkers that depend on enzymatic activity (e.g. induction of DNA repair enzymes may affect the level of DNA strand breaks). For other biomarkers a reduction in response compared with the control may not be possible or relevant but even in these cases a plateau level may be reached at high exposure concentrations. (figure 1, top panel).

Non-linear dose response relationships are not an indication of chaos or weakness due to experimental design or analytical techniques. Biology is seldom linear, but when we search for useful biomarkers for biomonitoring studies of oil/produced water discharges we look for a set of biomarkers responsive to environmentally relevant concentrations. Some biomarkers will not be sensitive enough to give a signal unless the animals are caged close to the platform or the fish have been swimming in the plume for several days. Other biomarkers may give a signal at low concentration, but may be inhibited at higher concentrations.

The possibility remains that detoxification and repair systems may be overwhelmed either by high and/or long term exposure, thus we may not get a biomarker signal, even if there is a risk of reduced fitness. It is important to be certain that this type of false negative is avoided when biomarkers are used in field studies.

Each biomarker and each species may have a window of response – a window in concentration and time where a biomarker signal can be detected. Some biomarkers

give a signal following only a few hours of exposure, others may need long term exposure to generate a signal. Some biomarkers are specific for certain compounds, such as PAH metabolites in the bile of fish. Other biomarkers are non-specific; alkaline unwinding and comet assay are non-specific genotoxicity biomarkers, while NRRT is a non-specific biomarker for cellular stress. Most experts in the biomarker field of research recommend a suite of biomarkers for biomonitoring.

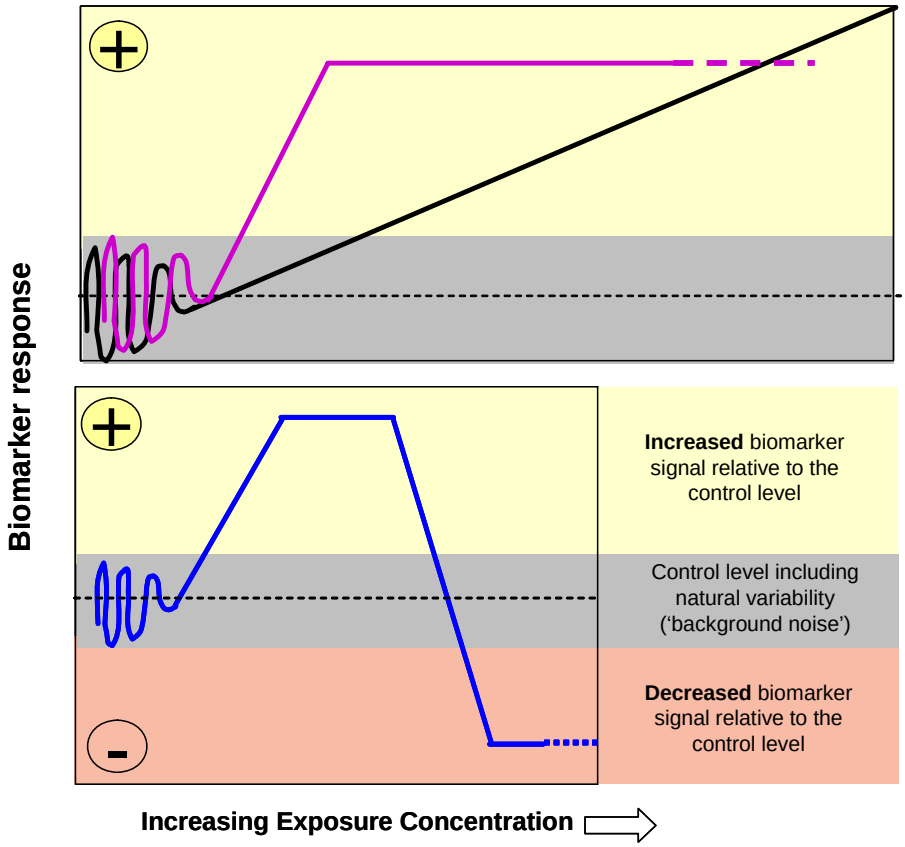

Figure 1. Theoretical linear (top panel) and non-linear (bottom panel) dose-response relationship for biomarker response. The grey area represents the natural variability around the control level. In the top panel the black graph represents a biomarker that gives an increasing signal with increasing concentration over a wide range of exposure concentrations. The pink graph represents a biomarker signal that reaches a plateau level above a certain exposure concentration (or tissue concentration). In the bottom panel the graph represents a biomarker signal that at low concentrations increase with increasing exposure concentration, reaches a plateau level at intermediate concentrations, followed by a lower biomarker signal than in the control (inhibition) at high concentrations. See text for more details.

2 Description of selected biomarkers

2.1 Genotoxicity tests

Aas *et al.* (2002) reported on the results from the genotoxicity tests performed in the POSCON experiments and Bechmann has written a literature review on results from genotoxicity tests used in the field (Bechmann, 2002). Based on the conclusions and recommendations in these reports we decided to apply the comet assay and the alkaline unwinding assay on the invertebrates (bivalves, sea urchins and shrimps), and to test a broader range of genotoxicity tests on cod.

Comet and alkaline unwinding assays both detect DNA strand breaks. In contrast to the Alkaline Unwinding assay the comet assay incorporates the microscopic examination of DNA damage to individual cell nuclei. The comet assay can detect DNA single strand breaks resulting from DNA damage, alkali labile sites and from excision repair. It is considered to be a sensitive, rapid and economic technique for the detection of strand breaks, which is ideally suited as a non-specific biomarker of genotoxicity in fish and other aquatic species (Mitchelmore and Chipman 1998). The DNA alkaline unwinding assay is a technique for measuring changes in the pattern of DNA unwinding during alkaline conditions. The unwinding of DNA from its double to single stranded form is dependent on the level of strand breaks already present, the more strand breaks, the faster unwinding.

Several literature reviews conclude that the DNA adduct levels in fish are effective molecular dosimeters of genotoxic contaminant exposure, e.g. Stein *et al.* (1994), Van Schooten *et al.* (1995), Qu *et al.* (1997) and Reichert *et al.* (1998). In organisms possessing active metabolic systems for a particular carcinogen, DNA adducts generally have longer biological half-lives than the substrate carcinogens. Thus, measurement of specific DNA adduct concentrations may provide a relevant biological indicator of prior exposure to environmental carcinogens (Van Schooten *et al.,* 1995).

The micronucleus test (MN) measures the extent of fragments detaching from the mitotic spindle. These displaced fragments or chromosomes enter the cytoplasm where they assume the morphology of minute micronuclei at the subsequent interphase.

2.2 Oxidative stress

In order to protect themselves against harmful radicals produced continuously in the partial reduction of molecular oxygen and other elements, aerobic organisms have evolved an antioxidant system. The complex system utilises a number of enzymes including **catalase**. During the metabolism of various inorganic and organic xenobiotic molecules (including PAHs), the generation of harmful radicals, such as active oxygen forms, is increased. Catalase catalyses the scavenging of hydrogen peroxide ($2H_2O_2 \rightarrow 2H_2O + O_2$) from the cell, and changes in catalase activity are used in biomonitoring to signal biological stress due to active oxygen species.

Glutathione S-transferase (GST) is part of the detoxification system and has evolved in organisms in order for them to be able to convert lipophlic compounds into more hydrophilic and thereby more excretable metabolites. Two major types of reactions exist: Phase I, which involves hydrolysis, oxidation and reduction, and Phase II, which involves conjugation or synthetic processes. GST catalyses one of these Phase II processes, the conjugation of glutathione to compounds with electrophilic centres. These electrophilic centres may otherwise be harmful as they may react with macromolecules controlling cell growth, such as DNA, RNA and proteins. It is therefore of great importance that the animal is capable of neutralising and excreting these compounds. Changes in the activity of GST may reflect exposure to xenobiotics.

The use of catalase and GST as biomarkers has been suggested in several marine organisms and although rarely dose-dependent, the utility of these enzymes lies in their sensitivity to reveal the onset of oxidative perturbation (Regoli *et al.,* 2004). Increased activities of catalase have been described for several fish and invertebrate species from polluted sites, but also inhibition and transitory responses are reported according to the intensity and duration of chemical disturbance. For GST, induction has been widely demonstrated following exposure to some organic contaminants (references in Regoli *et al.,* 2004), while the inhibition of this enzymatic activity has been indicated as a more specific response to chemical challenge (Regoli *et al.,* 2003).

The total oxyradical scavenging capacity (TOSC) assay measures the capability of the whole antioxidant system to neutralise various oxyradicals, quantifying the resistance of a biological tissue against these molecules. Regoli writes that while the analysis of individual antioxidants is useful for determining their sensitivity and to

understand the mode of action of a stressor, integration of the total antioxidant capacity provides a more holistic assessment of the overall biological significance of such variations (Regoli *et al.,* 2002). TOSC has a greater predictive value on the health condition of the organisms and allows discrimination of the different roles of specific ROS in the oxidative stress syndrome (Regoli *et al.,* 2002).

2.3 Neutral Red Retention Time (NRRT)

The Neutral Red Lysosomal Retention Assay measures contaminant-induced lysosomal membrane damage. The test makes use of the fact that only lysosomes in healthy cells can retain neutral red after initial uptake. Sub-cellular retention of neutral red is measured at timed intervals using light microscopy. Injured lysosomal membrane stability is thought to be a general measure of stress. Cells from animals exposed to environmental pollutants will exhibit reduced retention times for the probe compared to cells of animals from clean sites. The test has been developed by David Lowe for use on molluscs (Lowe *et al.*, 1992; Lowe *et al.*, 1995) and this method has been adapted for use on blood cells from shrimps and sea urchins here at Akvamiljø.

2.4 Additional fish biomarkers

The following biomarkers were analysed in oil exposed cod in the BioSea experiments: bile metabolites (analysed by fixed fluoresce equivalents and GC/MS), hematocrit, CYP1A, GST, VTG, ZRP, alkaline unwinding, Micronuclei, Comet assay, DNA adducts and histology of gills and liver. More biomarkers were studied in the oil exposed cod than in the invertebrates. For descriptions of hematocrit, Cyp1A, VTG, ZRP, and histology, see the report of A. Skadsheim.

3 Biomarker responses in oil exposed animals

In this chapter the results from statistical comparisons of biomarker responses in oil exposed animals and control animals in each of the laboratory experiments performed in BioSea JIP is presented within a set of tables and a short list of the main conclusions drawn for each of the tested biomarkers is provided. The recommendations given in the next chapter are based on the data presented here.

3.1 Genotoxicity tests

Tables 1 – 6 give the main results from statistical comparisons of results from genotoxicity tests on oil exposed animals and control animals.

3.1.1 Comet assay

- The Comet assay works well on blood-cells from blue mussels, Arctic scallops and green sea urchins, and on mussel embryos. The Comet assay has also worked well on sperm from sea urchins and mussels (The Poscon 4 experiment and Beep).
- The Comet assay needs further optimization for use on blood cells from cod, although it works well on blood cells from wrasse in our lab, and on several other species of fish tested in other labs.
- The Comet assay was tested on shrimp larvae and on blood cells, but without success.
- Statistically significant increases in the level of DNA strand breaks were observed in:
  - ..... blood cells from sea urchins exposed to oil concentrations in the range 4 – 1000 µg/L THC
  - …. blood cells from sea urchins exposed for 2 weeks to 1000 µg/l oil followed by 3 weeks recovery (Aas *et al.*, 2002)
  - …. sperm from sea urchins exposed to 85 µg/l THC (though the controls were variable)
  - ….. blood cells from mussels exposed to oil concentrations in the range 3 – 1000 µg/L THC
  - … blood cells from mussels exposed for 2 weeks to 1000 µg/l oil followed by 4 weeks recovery (Aas *et al*., 2002)
  - ….. 1-day old exposed mussel embryos from parents exposed for 7 months to 63 µg/l THC (larvae from other treatments were not tested)
  - …. sperm from mussels exposed to 63 µg/l THC (though the controls were variable)

- …. blood cells from Arctic scallops exposed to 14 and 64 µg/L THC. No significant response was observed in blood cells from scallops exposed to 2 µg/L THC

- … blood cells from cod exposed to 1000 µg/l oil in the Poscon 1 experiment, though there was high variability in the controls. Similar results were not obtained in cod blood cells sampled from the North Sea and Barents Sea exposures (Skadsheim, 2004)

- Comet assays of blood cells and sperm from mussels and sea urchins were used in the POSCON experiments and worked well on these species. Based on the long term exposures carried out in 2002 and 2003 it can be concluded that the Comet assay also works well on Arctic scallop and mussel larvae, but not on shrimps and cod. Comet has been used on shrimp embryos from the shrimp *Paleomonetes pugio* (Hook & Lee, 2004), but further method development/optimizing is needed to apply this method to the Northern shrimp. The Comet assay may not be a useful biomarker for oil exposure of shrimps, but since it works on the embryos of a different shrimp species, optimization of the method for the Northern shrimp embryos should be possible. Based on literature data using the Comet assay on several fish species (but not cod), and our experience from Comet assay on blood cells from wrasse, there is no reason that the Comet assay should not work well on blood cells from cod. It should be possible to solve the problem of high variability in the level of DNA strand breaks in blood cells from cod with further optimizing of the method. However, since two other genotoxicity tests appear to be able to detect DNA damage in oil exposed cod, it may be more important to invest further effort in optimizing the comet assay for use with invertebrate species.

3.1.2 Alkaline unwinding (AU)

- Alkaline unwinding worked well on hepatopancreas tissue from shrimps, particularly in the Statfjord exposure, showing a dose as well as time dependent response, indicating increases in the number of strand breaks with increased concentration and prolonged exposure. A statistically significant increase in DNA damage was detected by the AU assay in the hepatopancreas of shrimps exposed to oil concentrations in the range 4 – 1000 µg/L THC compared to controls.

- No increase in DNA damage measured by the AU assay was detected in mussels, Arctic scallops, sea urchins or cod exposed to oil. It is likely that the AU assay will be more sensitive with use of more replicate samples and further optimization of the method for these species.

- The alkaline unwinding assay has been able to distinguish polluted sites from reference sites (fish and crustaceans), but it has not been used in oil/produced water contaminated sites.

- Appendix 1 shows that AU can be a good biomarker for species in addition to shrimps.

3.1.3 Micronuclei (MN)

- The MN test worked well on cod liver cells.

- For the North Sea cod (exposed to Statfjord B oil) time and exposure dose dependent responses in MN frequencies were recorded at all exposure concentrations. Significantly more micronuclei in North Sea cod exposed to 482 µg/L THC (oil) were detected. There was a tendency to more micronuclei in North Sea cod exposed to lower concentrations.

- The MN frequencies in Barents Sea cod exposed to Goliat oil generally increased later in the experiment and at higher oil doses than in the North Sea cod exposed to Statfjord oil. In the Barents Sea cod recovery was slower and most MN was in fact found at the end of the recovery period. There were significantly more micronuclei in Barents Sea cod exposed to 1000 µg/L THC + spike[1].

- Tendency to more micronuclei in Barents Sea cod than in the control fish at lower concentrations.

- Significantly more micronuclei in gill cells from mussels exposed to 1000 µg/l oil, but the MN-test was not used at lower concentrations of oil.

1 Spike: A mixture of PAHs (186 µg/L) and a mixture of alkylated phenols (203 µg/L) was added to 1000 µg/L oil . See appendix 3 in Skadsheim *et al.* (2005) for composition of the PAH and AP mix.

- The micronucleus test has not been used on shrimps and sea urchins in BioSea. We have not found references to use of the MN test on any tissue in adult sea urchins, but Saotome *et al.* (1999, 2003) have developed a micronucleus test for early blastula (embryos) of the two sea urchin species *Clypeaster japonicus* and *Hemicentrotus pulcherrimus,* It is possible that this test can be used also on *S. droebachiensis* embryos. Janina Barsiene tried the MN test on different tissues from crabs, but did not find enough dividing cells. We have not been able to find any other reference on MN in crustaceans. It may however, be possible to do MN on shrimp embryos, since cells are dividing more rapidly in embryos than in adult animals.

- Pilot studies with edible crab *(Cancer pagurus)* indicated that the tested tissues (hepatopancreas, green gland and gonad) were unsuitable for the micronuclei assay (Aas *et al.*, 2002).

3.1.4 DNA adduct

- All exposed groups of North Sea cod and Barents Sea cod had higher levels of DNA adducts in the liver than the corresponding control fish in the BioSea JIP cod exposures (Appendix 6). Generally the level of DNA adducts increased with increasing exposure concentration of oil, tissue concentration of PAHs and the level of PAH metabolites in the bile. For more details see Skadsheim's BioSea JIP fish report (Report AM 2004/005). The measured concentration of THC in the lowest concentration tested in these exposures was 43 µg/L THC (which was similar to in Aas *et al.*, 2000).

- The DNA adduct assay test works well on fish. Significantly higher levels of DNA adducts have been observed in cod exposed to 40 µg/L oil (Aas *et al.*, 2000, Skadsheim, 2005), sheepshead minnows exposed to 100 µg/L oil (Bechmann *et al.*, 2004), and to 2 µg/L PAH-mix (DREAM) (Beyer *et al.*, 2004). In the BioSea experiments the invertebrates were exposed to lower levels of oil than the cod. It is important to test the sensitivity of the DNA adduct analysis at even lower oil concentrations.

- *Results from relevant biomonitoring study:* Feral haddock collected in the Tampen area in the North Sea had significantly more PAH metabolites in bile and more DNA adducts in the liver than haddock from the reference site (Klungsøyr *et al.)*

- No increase in DNA adducts was observed in mussels exposed to 1000 µg/l oil. The test was not used on molluscs at lower concentrations of oil.
- There were significantly more DNA adducts in sea stars exposed to 1400 µg/L oil, but this test has not been used on echinoderms at lower concentrations of oil (POSCON 1; Taban *et al.*).
- The DNA adduct test has not been used on sea urchins, Arctic scallops or shrimps in the BioSea exposures.

Table 1. Results from statistical analyses of the differences in genetic damage between oil exposed and control **molluscs** in the BioSea JIP experiments. Comparison of the 4 different genotoxicity tests applied on invertebrates in the BioSea JIP program. Relevant data from the POSCON experiments are included (Taban *et al.,* 2002, Aas *et al.,* 2002).

+++ Significant increase in DNA damage compared to corresponding control level ($p<0.05$)
n.s.: No statistically significant difference from the control level ($p<0.05$)

Species	Tissue	Exposure	Conc.	Exposure time	Genotoxicity test			
					Alkaline unwinding	Comet assay	Micronuclei	DNA adducts
Blue Mussel *Mytilus edulis*	Gill	POSCON 1 North Sea Oil	1400 µg/L oil	2 weeks	*not tested*	*not tested*	*not tested*	**n.s.**
	Haemocytes	POSCON 4 North Sea oil	1000 µg/L oil			+++	**n.s.**	*not tested*
	Sperm						*not tested*	
	Digestive gland				**n.s.**	*not tested*	+++	
	Gill							
	Haemocytes		(1000 µg/L oil)	4 weeks depuration	*not tested*	+++	*not tested*	
	Haemocytes	North Sea oil	3, 15, 63 µg/L THC	1 month		+++		
	Haemocytes			7 months		+++		
	Sperm				(but gonads tested)	+++ for 63 µg/L		
	Gonad				**n.s.**	(but sperm tested)		
	Embryo		63 µg/L THC	parental transfer	*not tested*	+++		
Arctic scallop *Chlamys islandica*	Haemocytes	Goliath oil	2,14, 64 µg/L THC	1 month		+++ for 14 and 64 µg/L		
	Gills				**n.s.**	*not tested*		

Table 2. Results from statistical analyses of the differences in genetic damage between oil exposed and control **shrimps** in the BioSea JIP experiments. Comparison of the 4 different genotoxicity tests applied on invertebrates in the BioSea JIP program. Relevant data from the POSCON experiments are included (Taban *et al.,* 2002, Aas *et al.,* 2002).

+++ Significant increase in DNA damage compared to corresponding control level ($p<0.05$)

n.s.: No statistically significant difference from the control level ($p<0.05$)

Species Tissue	Exposure	Conc.	Exposure time	Genotoxicity test			
				Alkaline unwinding	Comet assay	Micronuclei	DNA adducts
Northern Shrimp *Pandalus borealis* Hepatopancreas	POSCON 4 North Sea oil	1000 µg/L oil	2 weeks	**+++**	*(not successful on 'blood' and larvae)*	*not tested*	*not tested*
	North Sea oil	4, 21, 90 µg/L THC	1 month	**+++ for 90 µg/L**			
		90 µg/L THC	3 months	**+++**			
		4, 21 µg/L THC	5 months	**+++**			
	Goliath oil	5, 23, 102 µg/L THC	1 month	**+++ for 102 µg/L**			

Table 3. Results from statistical analyses of the differences in genetic damage between oil exposed and control **echinoderms** in the BioSea JIP experiments. Comparison of the 4 different genotoxicity tests applied on invertebrates in the BioSea JIP program. Relevant data from the POSCON experiments are included (Taban *et al.,* 2002, Aas *et al.,* 2002).

+++ Significant increase in DNA damage compared to corresponding control level ($p<0.05$)

n.s.: No statistically significant difference from the control level ($p<0.05$)

Species	Tissue	Exposure	Conc.	Exposure time	Genotoxicity test			
					Alkaline unwinding	Comet assay	Micronuclei	DNA adducts
Green Sea urchin *Strongylocentrotus droebachiensis*	Coleomocytes	POSCON 2 North Sea oil	1000 µg/L oil	2 weeks	*not tested*	+++	*not tested*	*not tested*
		North Sea oil	29, 85 µg/L THC	1 month		+++		
			4, 29, 85 µg/L THC	4 months				
				7 months		+++		
	Sperm			7 months	(but gonads tested)	+++ for 85 µg/L		
	Gonad				**n.s.**	(but sperm tested)		
	Coleomocytes	POSCON 4 North Sea oil	1000 µg/L oil	2 weeks	*not tested*	significant differences		
	Sperm							
	Coleomocytes		(1000 µg/L oil)	3 weeks depuration				
Starfish *Asterias rubens*	Digestive gland	POSCON 1 North Sea Oil	1400 µg/L oil	2 weeks	*not tested*	*not tested*	*not tested*	+++

Table 4. Results from statistical analyses of the differences in genetic damage between oil exposed and control **fish** in the BioSea JIP experiments. Comparison of the 4 different genotoxicity tests applied on invertebrates in the BioSea JIP program. Relevant data from the POSCON experiments are included (Taban *et al.,* 2002, Aas *et al.,* 2002).

+++ Significant increase in DNA damage compared to corresponding control level (p<0.05)
n.s.: No statistically significant difference from the control level (p<0.05)

Species		Tissue	Exposure	Conc. oil	Exposure time	Genotoxicity test			
						Alkaline unwinding	Comet assay	Micronuclei	DNA adducts
North Sea Cod	*Gadus morhua*	Blood	POSCON 1 North Sea oil	1400 µg/L	2 weeks	+++	+++	*Not tested*	
		Liver	POSCON 4 North Sea oil	1000 µg/L	2 weeks	+++	*not tested*	+++	+++
		Erythrocytes Head Kidney				*not tested*		+++	*not tested*
		Erythrocytes caudal vein						n.s.	
		Erythrocytes gills							
		Spleen							
		Liver	North Sea oil	43, 112, 482 µg/L	day 3, 14, 24 recovery: day 27, 38	n.s.		+++ for 482 µg/L tendencies at lower conc.	+++[1)]
Barents Sea Cod			Goliath oil	60, 250, 1000 µg/L spike+1000 µg/L (nom.)	day 3, 17, 31 recovery: day 34, 45			+++ for 1000 µg/L + spike tendency at lower conc.	
		Blood cells		1000 µg/L	day 31	*not tested*	n.s.	*not tested*	*not tested*

[1)] DNA adducts were analysed in liver samples from 3 fish at each sampling time/treatment. Data from the last exposure day (ca 1 month) and the third day of the depuration period was combined and pair wise comparisons of the treatment groups was done using the non-parametric Wilcoxon test (p<0.05) (See Appendix 6).

Table 5. Results from statistical analysis of the differences **in Micronuclei frequency** in liver cells between North Sea Cod exposed to Statfjord B oil and Barents Sea cod exposed to Goliat oil compared to the activity level in the corresponding controls in the BioSea JIP experiments.

p = significance level : significantly increased frequency of MN
p = significance level; tendency to increased frequency of MN
n.s.: not significant (p>0.2)

Species Tissue	**Oil**	**Exposure time**	**Oil concentration:**			
			60 µg/l	**250 µg/l**	**1000 µg/l**	**1000 µg/l + spike**
Atlantic cod *Gadus morhua* Liver	Statfjord	3 days	**n.s.**	**n.s.**	p = 0.055	**n.s.**
		14 days	**n.s.**	p = 0.055	p = 0.008	p = 0.008
		24 days	**n.s.**	**n.s.**	p = 0.008	p = 0.016
		Recovery – 3 days	**n.s.**	**n.s.**	p = 0.031	p = 0.15
		Recovery – 14 days	**n.s.**	**n.s.**	p = 0.008	p = 0.2
Barents Sea Cod *Gadus morhua* Liver	Goliat	3 days	**n.s.**	**n.s.**	**n.s.**	p = 0.055
		17 days	**n.s.**	**n.s.**	**n.s.**	p = 0.055
		31 days	p = 0.095	**n.s.**	**n.s.**	p = 0.008
		Recovery – 3 days	**n.s.**	**n.s.**	**n.s.**	p = 0.0317
		Recovery – 14 days	**n.s.**	**n.s.**	**n.s.**	**n.s.**

Table 6. Results from statistical analysis of the differences in genetic damage between oil exposed and control animals in the BioSea JIP experiments. The oil concentrations were in the range 2-5 µg/l THC ('**Low**'), 14-43 µg/l THC (60 µg/l oil nominal for the fish) ('**Medium**'), and 63-102 µg/l THC (250 µg/l oil nominal for the fish) ('**High**'). The grey boxes signify that we do not have data for this concentration/exposure time.

+++ Significant increase in DNA damage compared to corresponding control level (p<0.05)
\+ Increased DNA damage, but not statistically significant (p>0.05)
n.s. The difference between control and exposed was not statistically significant at the p<0.05 level.

Species Tissue	**µg/L THC** (oil)	**Expo. time**	**Genotoxicity test**	**Concentration of oil**		
				Low	**Medium**	**High**
Blue Mussel Haemocytes	Statfjord 3, 15, 63	1 month	**Comet**	+++	+++	+++
		7 months		+++	+++	+++
Arctic scallop Haemocytes	Goliat 2,14, 64	1 month		**n.s.**	+++	+++
Northern Shrimp Hepatopancreas	Statfjord 4, 21, 90	1 month	**Alkaline unwinding**	**n.s.**	**n.s.**	+++
		5 (3) months		+++	+++	+++
	Goliat 5, 23, 102	1 month		**n.s.**	**n.s.**	+++
Sea urchin Coelomocytes	Statfjord 4, 29, 85	1 month	**Comet**		+++	+++
		4 months		+++	+++	+++
		7 months		+++	+++	+++
North Sea Cod Liver	Statfjord 43 and 112	24 days	**DNA adducts**		+++[2)]	+++[2)]
			Micronuclei		**n.s.**	+ p=0.1
Barents Sea Cod Liver	Goliat 60 and 250[1)]	31 days	**DNA adducts**		+++[3)]	+++[3)]
			Micronuclei		+ p=0.1	**n.s.**

[1)] Nominal concentration
[2)] Comparisons based on combined data from day 24 and 27.
[3)] Comparisons based on combined data from day 31 and 34.

3.2 Oxidative stress parameters

Tables 7 – 8 give the main results from statistical comparisons of results from oxidative stress parameters analysed in oil exposed animals and control animals.

3.2.1 Glutathione-S-Transferase (GST) activity

Increased GST activity in exposed animals indicate an increase in the animals capacity to detoxify and excrete harmful substances.

- After one month exposure to 63 µg/l THC the GST level in the hepatopancreas of mussels was significantly higher than in the controls (Baussant, 2004).
- Increased GST activity in sea urchins exposed to 85 µg/l THC for 1 month, but the activity was not significantly higher than that measured in the control.
- One month exposure of Arctic scallops (*Chlamys islandica*) to 2, 14 and 64 µg/l Goliath oil caused increased activity of GST, but it was only the activity in the hepatopancreas from animals exposed to the lowest concentration that was significantly different from the control.
- Significantly increased GST activity was measured in North Sea cod exposed to 250 µg/L Statfjord B oil and higher at day 3, but no change in GST activity measured on day 14, and a response only found at the highest concentrations on day 24. No significant change in the GST activity was observed in North Sea cod exposed to 60 µg/l Statfjord oil for 3, 14 or 24 days.
- A significant increase in GST activity was observed in Barent Sea cod following 3 days exposure to 60 µg/L Goliat oil and 1000 µg/L + spike. A reduced response was observed at intermediate concentrations and following longer exposure period (17 and 31 days).

Decreased GST activity levels indicate a direct toxic effect of the enzyme itself (inactivation) or possibly a more general stress effect in the animal.

- Oil concentrations in the range 4-90 µg/l THC caused a significantly lower level of GST activity in the hepatopancreas of shrimps than in the control shrimps after 5 (3) months exposure

- Sea urchins exposed to 1.4 mg/l oil in the POSCON 2001 experiment showed a decrease in GST activity (The POSCON 1 experiment, Taban *et al.*, 2002).

- Indications of decreased GST activity were observed in sea urchin gut following 7 months exposure to 29 and 85 µg/l THC, but the differences were not statistically significant.

3.2.2 Catalase activity

Increased catalase activity indicates that the test animal has an increased ability to respond to oxidative stress

- The dose-response relationship for catalase activity with increasing oil concentration was bell-shaped for *Chlamys* exposed to Goliat for 1 month, shrimps exposed to North Sea oil for 5 (3) months and sea urchins exposed to North Sea oil for 7 months. Statistically significant increases in catalase activity were recorded at 2, 14 and 64 µg/l for *Chlamys*, 29 µg/l for sea urchin and 21 µg/l for shrimps.

Decreased catalase activity levels indicate a direct toxic effect of the enzyme itself (inactivation) or possibly a more general stress effect in the animal

- The catalase activity in shrimps exposed to Goliat oil was significantly decreased after 1 month exposure to 102 µg/l THC.

3.2.3 Total Oxyradical Scavenging Capacity (TOSC)

Increased level of TOSC indicate increased capacity to remove radicals and reduced oxidative stress.

- The level of TOSC against peroxyl radical (ROO $^{\cdot}$) was bell-shaped for scallops exposed to Goliat oil. TOSC was significantly higher in scallops exposed to 2 and 14 µg/l Goliat oil for 1 month than in the control.

- TOSC against hydroxyl radicals (OH $^{\cdot}$) was higher in all exposed groups after 1 month exposure to Goliat oil, but the difference from the control was only significant at the lowest and the highest exposure (4.5 and 102 µg/l).

Decreased levels of TOSC indicate higher susceptibility to oxidative stress.

- The level of TOSC against peroxyl radical (ROO) was significantly lower in shrimps exposed to 90 µg/l Statfjord oil for 1 month, but no response was observed in shrimps exposed to Goliat oil for 1 month.

- The TOSC (ROO) level was lower in all exposed groups after 3/5 months exposure to Statfjord oil, but the difference from the control was only significant at the lowest and the highest exposure (4 and the 90 µg/l).

Table 7. Results from statistical analysis of the differences in **Glutathione-S-Transferase** (**GST) and Catalase activity** and the **Total Oxygen Scavenging Capacity (TOSC)** between oil exposed and control animal in the BioSea JIP experiments.

+++	Significantly increased activity/capacity compared to corresponding control level
- - -	Significantly reduced activity/capacity compared to corresponding control level
+	Increase activity/capacity, but not statistically significant (p>0.05)
-	Reduced activity/capacity, but not statistically significant (p>0.05)
n.s.	The difference between control and exposed was not statistically significant at the p<0.05 level.

Species Tissue	**µg/L THC** (oil)	**Exposure time**	**Biomarker**	**Oil concentration** x)		
				Low	**Medium**	**High**
Blue Mussel *Mytilus edulis* Hepatopancreas	Statfjord oil 3, 15, 63	1 month	**GST**	+ n.s.	n.s.	+++
			Catalase	+ n.s.	n.s.	+ n.s.
			TOSC (ROO·)	+ n.s.	+ n.s.	+ n.s.
		7 months	**GST**	- n.s.	- n.s.	+ n.s.
			Catalase	n.s.	n.s.	+ n.s.
			TOSC (ROO·)	+ n.s.	- n.s.	+ n.s.
Arctic scallop *Chlamys islandica* Hepatopancreas	Goliat oil 2,14, 64	1 month	**GST**	+++	+ n.s.	+ n.s.
			Catalase	+++	+++	+++
			TOSC (ROO·)	+++	+++	+ n.s.
Northern Shrimp *Pandalus borealis* Hepatopancreas	Statfjord oil 4, 21, 90	1 month	**GST**	- n.s.	n.s.	+ n.s.
			Catalase	+ n.s.	+ n.s.	+ n.s.
			TOSC (ROO·)	- n.s.	+ n.s.	- - -
		5 (3) months	**GST**	- - -	- - -	- - -
			Catalase	+ n.s.	+++	+ n.s.
			TOSC (ROO·)	- - -	- n.s.	- - -
			TOSC (OH·)	too high variability		
	Goliat oil 5, 23, 102	1 month	**GST**	+ n.s.	+ n.s.	n.s.
			Catalase	n.s.	n.s.	- - -
			TOSC (ROO·)	+ n.s.	+ n.s.	n.s.
Green Sea urchin *Strongylocentrotus droebachiensis* Gut	Statfjord oil 4, 29, 85	1 month	**GST**	Not analysed		+ n.s.
			Catalase	Too low activity		
			TOSC	Not analysed		
		7 months	**GST**	- n.s.	- n.s.	- n.s.
			Catalase	+ n.s.	+++	+ n.s.
			TOSC	Not analysed		

Table 8. Results from statistical analysis of the differences in **Glutathione-S-Transferase** (**GST) activity** between North Sea Cod exposed to Statfjord B oil and Barents Sea cod exposed to Goliat oil compared to the activity level in the corresponding controls in the BioSea JIP experiments.

p = significance level : significantly increased enzyme activity
p = significance level; tendency to increased GST activity
p = significance level; tendency to reduced GST activity
n.s. no statistically significant difference in GST activity between control and oil exposed groups (p>0.2)

Species Tissue	**Oil**	**Exposure time**	**Oil concentration:**			
			60 µg/l	**250 µg/l**	**1000 µg/l**	**1000 µg/l + spike**
Atlantic cod *Gadus morhua* Liver	Statfjord	3 days	**n.s.**	p = 0.036	p = 0.023	p = 0.06
		14 days	**n.s.**	**n.s.**	**n.s.**	**n.s.**
		24 days	**n.s.**	p = 0.14	p = 0.036	p = 0.012
		Recovery – 3 days	**n.s.**	**n.s.**	**n.s.**	p = 0.036
		Recovery – 14 days	**n.s.**	p = 0.012	p = 0.021	p = 0.012
Barents Sea Cod *Gadus morhua* Liver	Goliat	3 days	p = 0.02	**n.s.**	p = 0.17	p = 0.036
		17 days	**n.s.**	p = 0.09	p = 0.14	**n.s.**
		31 days	**n.s.**	**n.s.**	**n.s.**	**n.s.**
		Recovery – 3 days	**n.s.**	**n.s.**	**n.s.**	p = 0.016
		Recovery – 14 days	**n.s.**	**n.s.**	p = 0.021	p = 0.028

3.3 Neutral Red Retention Time (NRRT)

Table 9 gives the main results from statistical comparisons of results from the NRRT test on oil exposed and control invertebrates.

- NRRT worked well in both shrimp exposures and showed dose dependent as well as time dependent responses, indicating lowered lysosomal membrane stability and therefore an impaired immune system. There were statistically significant differences between the control and the 21 and 90 µg/L THC exposed shrimps in the Statfjord experiment, and in all treatments (5, 23, 102 µg/L) in the Goliat exposure.

- Decreasing membrane stability with increasing oil concentration in the range 4 – 85 µg/l indicated that the sea urchins were stressed, although the difference was not statistically significant at the lowest exposure concentration.

- Decreasing membrane stability with increasing oil concentration was observed for Arctic scallops exposed to 2-64 µg/l THC (Goliat oil) and sea urchins exposed to 4-85 µg/l THC (Statfjord B oil), indicating that the animals were stressed, although the difference was not statistically significant at the lowest exposure concentration.

- There was a relatively large spread of values in the distribution of NRRT in mussel haemocytes. Hence, despite a clear reduction of the median NRRT at 3 µg/L THC after 1 month, there was no statistically significant difference. After 7 months exposure, the blood of a large number of the control mussels was contaminated by micro-organisms. Hence, the test could not be validated. In previous oil exposure experiments with mussels significant differences have been observed with the NRRT assay between controls and mussels exposed to 500 and 1000 µg/L oil (Beep and Poscon 4).

Table 9. Results from statistical analysis of the difference in **lysosomal membrane stability (NRRT)** between oil exposed and control animal in the BioSea JIP experiments.

p<0.05: Oil exposed animals have significantly lower lysosomal membrane stability than control animals
n.s.: no statistically significant difference from the control level
(p = x : lower lysosomal stability than in control, but p>0.05)

Species	Exposure µg/l THC	Exposure time	Oil concentration		
			Low 2-5 µg/l	Medium 14-29 µg/l	High 63-102 µg/l
Blue Mussel *Mytilus edulis*	Statfjord oil 3, 15, 63	1 month	**n.s.**	**n.s.**	**n.s.**
		7 months	**n.s.**	**n.s.**	**n.s.**
Arctic scallop *Chlamys islandica*	Goliat oil 2,14, 64	1 month	**n.s.**	p<0.05	p<0.05
Northern Shrimp *Pandalus borealis*	Statfjord oil 4, 21, 90	1 month	**n.s.**	**n.s.**	p<0.001
		5 (3) months	**n.s.**	p<0.001	p<0.0001
	Goliat oil 5, 23, 102	1 month	**n.s.**	p<0.0001	p<0.0001
		3.5 months	p = 0.014	p<0.001	p<0.0001
Green Sea urchin *Strongylocentrotus droebachiensis*	Statfjord oil 4, 29, 85	1 month	p = 0.27	p = 0.23	p=0.003
		4 months	p = 0.16	p = 0.0025	p = 0.0033

4 Recommended biomarkers

Table 10 and 11 give a summary of all significant biomarker responses in the BioSea JIP experiments with increasing exposure concentration and time for invertebrates and fish. Generally, an increasing number of biomarkers gave statistically significant responses with increasing oil concentration for invertebrates and fish. But there are exceptions to this general trend, e.g. oxidative stress in scallops.

For the biomarkers that were measured in invertebrates following 1 month and 3-5 months exposure, more significant responses were detected with increasing exposure time. But again there are exceptions to the general trend. There was a tendency to higher

micronuclei frequency in cod liver with increasing exposure time, but this trend was not as evident for GST and Cyp1A.

The nominal concentration of 60 µg/l for cod corresponds to the medium concentration for invertebrates. Generally less significant biomarker responses were detected in fish than in invertebrates at the same exposure concentration of oil. In previous experiments where fish have been exposed to oil significant increase in the level of PAH metabolites in bile and DNA adducts in the liver has been detetcted (Aas *et al.*, 2000). In the present BioSea experiment there was a significant increase in the level of DNA adducts in the liver of cod exposed to all tested concentrations of Statfjord B oil and Goliat oil (60 - 1000 µg/L).

The results from the lowest oil exposure in the invertebrate experiments indicate that biomarkers are useful tools for biomonitoring even at this low concentration, though none of the selected biomarkers gave a significant signal at this concentration following 1 month exposure. This has to be kept in mind when biomonitoring studies are planned. It is possible that 1 month was too long – or not long enough – for some of the biomarker signals, or that more replicates or further optimization of the various methods will make it easier in the future to detect oil exposures even lower than those tested in BioSea by use of biomarkers.

Generally we recommend the same set of biomarkers for biomonitoring of the North Sea and the Barents Sea. There were more similarities than differences in the biomarker signals in the two series of exposures. Observed differences in biomarker responses in molluscs, shrimps and fish between the ’North Sea’ and the ’Barents Sea’ exposures, could be due to temperature and type of oil, and additionally, two species of molluscs and two strains of cod were used.

In the Barents Sea there will not be a continuous discharge of produced water (PW) as in the North Sea. Monitoring of caged and wild organisms can be carried out in the Barents Sea to ensure compliance of the zero discharge agreement between regulators and the oil industry. Hence no changes in biomarker responses are to be expected, even very close to the installations.

In the North Sea, biomarkers can be used for biomonitoring of PW discharges. Relatively large amounts of PW are discharged, and significant biomarker responses can

be expected in animals caged close to the platforms. Cages with animals for biomonitoring can be placed out in a transect from platforms to monitor that no harmful discharges occur. The most suitable organisms for caging of those tested in BioSea project are the blue mussels (North Sea), Arctic scallops (Barents Sea) and cod. Based on the study by Krause *et al.* (1994) caging of sea urchins is possible, but in the study of Krause urchins were caged on the sea floor exposing them both to contaminants in the sediments and in the water. *S. droebachiensis* is a relevant species for both the Barents Sea and the North Sea. Shrimps are ecologically and economically important. We suggest that a pilot study is conducted to evaluate the suitability of using caged shrimps in biomonitoring studies. In general a minimum of three stations along a transect is recommended, and it is important to sample a sufficient number of individuals to ensure statistical robustness. The provision of good reference stations is also crucial to the process. If the animals at the reference station are in bad condition, for whatever reason, it will be difficult to conclude anything from biomarker responses analysed in animals caged/collected at the more exposed sites. Biomarkers can also be used for biomonitoring following an accidental (oil) spill in both areas (the Barents Sea and the North Sea). Caging of animals for biomonitoring can be carried out after an accidental oil spill at sites where the spill is likely to pass. Modelling of current and wind direction etc. can be used to decide where to place cages and sample wild organisms to find out how large area has been affected and to study recovery. Collection of feral cod, shrimps and Arctic scallops (Barents Sea) along a transect from oil installations, or at different distances from an oil spill, is also a relevant biomonitoring strategy.

We recommend the use of more than one species and a suite of biomarkers for biomonitoring PW discharges. In shrimps alkaline unwinding and NRRT appeared to be sensitive biomarkers for exposure to oil. Comet assay and NRRT appeared to be sensitive biomarkers for exposure to oil in blue mussels and sea urchins. The results from analysis of oxidative stress biomarkers were less conclusive for shrimps, sea urchins and blue mussels. Oxidative stress parameters were, however, more sensitive to low concentrations of oil for Arctic scallops. Higher concentrations of oil caused less response on GST and TOSC in scallops, but more response at the level of DNA strand breaks measured by the Comet assay. Catalase was however induced at all exposure concentrations in the Arctic scallops and we recommend catalase as a biomarker for biomonitoring in addition to the comet assay and NRRT. It has to be kept in mind that

NRRT and the comet assay have to be preformed on fresh (not frozen/fixed) cells. All the other biomarkers tested in the BioSea experiments can be analysed in frozen or fixed samples.

The optimum biomarkers for cod, based on the BioSea project and previous oil exposure experiments performed at Akvamiljø, are PAH metabolites in the bile, DNA adducts and MN frequency in the liver. DNA adducts have first priority as genotoxicity biomarker. Results from Cyp1A and GST analyses are presently less conclusive, but can also be recommended for biomonitoring of oil exposure in fish to gain more experience. Biomarkers other than those giving significant responses in the BioSea oil exposures may be relevant for biomonitoring because the off shore PW discharge may contain traces of chemicals and have a different composition than the oils tested in BioSea. Although no significant responses were detected in the VTG/ZRP assay in the BioSea experiments they are useful biomarkers for monitoring the presence of endocrine disrupters in discharges. It is likely that histopathological changes in the liver (and other tissues) of fish need longer than the 1 month exposure used in BioSea to develop. Histology may however be a useful parameter in wild caught fish. Analyses for alkylphenol metabolites in fish bile is being developed at Akvamiljø, and may also be a useful biomarker for monitoring of PW discharges.

Compared to the extensive set of biomarkers tested in the BECPELAG, Troll (Børseth & Tollefsen, 2004), and Tampen studies (Klungsøyr *et al.)* (appendix 5) a very limited set of parameters were tested in the BioSea exposures. When planning biomonitoring studies recommendations from these biomonitoring studies must be taken into consideration. Based on these studies GST in mussels and fish may be a useful biomarker for biomonitoring. The BioSea JIP studies did not clearly confirm this, although parts of the results were positive. Analysis of fatty acid composition and indicators of lipid peroxidative stress in fish also appear to hold promise (Klungsøyr *et al*.). The analysis of fatty acid composition of shrimp embryos (analysed by scientists at the Danish Technical University), and the analysis of neutral lipid accumulation and peroxisome proliferation in mussel gonad (analysed by scientists at the University of Bilbao, Spain) in animals from the BioSea exposures are also promising biomarkers for oil exposure.

When a suite of biomarkers is used for biomonitoring to cover a larger "window" of exposure conditions/sensitivity, the following question is important: Will response in *any* of the selected biomarkers be considered an early warning signal, or do we need response in more than one biomarker? (figure 2).

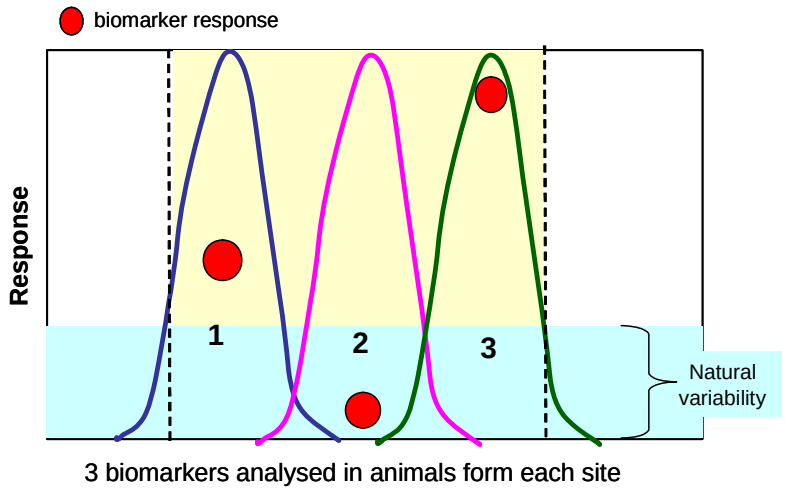

Figure 2. Interpretation of results from biomonitoring studies with a suite of biomarkers. The numbers 1, 2 and 3 refers to 3 biomarker response curves. Each biomarker may be specific to the type of discharge, the exposure time and the exposure concentration. The red circles are the biomarker responses. Biomarker 1 give a relatively small, but significant response, biomarker 2 does not give a significant response, but biomarker 3 give a high signal. If these were the results from a field monitoring study, how do we use the results when only one or two of the biomarkers respond?

Table 10. Statistical significance of biomarker responses in invertebrates with increasing exposure time and exposure concentration. Only biomarkers with statistically significant ($p<0.05$) responses are included in the table below. The selected biomarkers are glutathione-S-transferase (GST), catalase (CAT), total oxygen scavenging capacity (TOSC), comet assay, alkaline unwinding (AU) and lysosomal membrane stability (NRRT) tested in Northern shrimps *(Pandalus borealis),* blue mussels *(Mytilus edulis),* and green sea urchins *(Strongylocentrotus droebachiensis)* exposed to Statfjord B oil (S), and Northern shrimps and Arctic scallops *(Chlamys islandica)* exposed to Goliat oil (G). Biomarker followed by + means significantly increased activity/damage, and biomarker followed by – means significantly reduced/inhibited response compared to the control level.

Exposure time	Oil concentration (µg/l THC)		
	2-5 µg/l	14-29 µg/l	63-102 µg/l
1 month	GST+ (scallop - G) CAT+ (scallop - G) TOSC+ (scallop - G) Comet+ (mussel - S)	CAT+ (scallop - G) TOSC+ (scallop - G) Comet+ (mussel - S) Comet+ (scallop - G) Comet+ (sea urchin - S) NRRT + (scallop - G) NRRT + (shrimp - G)	GST+ (mussel - S) CAT+ (scallop - G) CAT- (shrimp - G) TOSC - (shrimp - S) Comet+ (mussel - S) Comet+ (scallop - G) Comet+ (sea urchin - S) AU+ (shrimp - S) AU+ (shrimp - G) NRRT + (scallop - G) NRRT + (shrimp - S) NRRT + (shrimp - G) NRRT + (sea urchin - S)
3-5 months	GST - (shrimp - S) TOSC - (shrimp - S) Comet+ (sea urchin - S) AU+ (shrimp - S) NRRT + (shrimp - G)	GST - (shrimp - S) Comet+ (sea urchin - S) AU+ (shrimp - S) NRRT + (shrimp - G) NRRT + (sea urchin - S) NRRT + (shrimp - S)	GST - (shrimp - S) TOSC - (shrimp - S) Comet+ (sea urchin - S) AU+ (shrimp - S) NRRT + (shrimp - S) NRRT + (shrimp - G) NRRT + (sea urchin - S)
7 months	Comet+ (mussel - S) Comet+ (sea urchin - S)	CAT+ (sea urchin - S) Comet+ (mussel - S) Comet+ (sea urchin - S)	Comet+ (mussel - S) Comet+ (sea urchin - S)

Table 11. Statistical significance of biomarker responses in cod with increasing exposure time and exposure concentration. Only biomarkers with statistically significant ($p<0.05$) responses are included in the table below. The selected biomarkers are glutathione-S-transferase (GST), miconuclei (MN) and Cyp1A for cod *(Gadus morhua)* exposed to Statfjord B oil (S) and Goliat oil (G). (Biomarker + means significantly increased activity/damage). Statistically significant responses were not detected in hematocrit, alkaline unwinding, comet, VTG and ZRP. Skadsheim will do statistical comparisons of the results from the analysis of PAH metabolites in the bile for the planned publications. The trends show increasing concentrations of bile metabolites with increasing exposure concentration.

Exposure time	Nominal oil concentration (µg/l)			
	60	250	1000	1000 + spike
3 days	GST + (G)	GST + (S) Cyp1A+ (S)	GST + (S) Cyp1A+ (S) Cyp1A+ (G)	GST + (G) Cyp1A+ (S)
14/17 days		Cyp1A+ (S)	MN+ (S) Cyp1A+ (S)	MN+ (S) Cyp1A+ (S) Cyp1A+ (G)
24/31 days		Cyp1A+ (S) Cyp1A+ (G)	MN+ (S) GST + (S) Cyp1A+ (S) Cyp1A+ (G)	MN+ (S) MN+ (G) GST + (S) Cyp1A+ (S) Cyp1A+ (G)
DNA adduct data from last exposure day and third day of recovery[1)]	DNA adducts + (S,G)	DNA adducts + (S,G)	DNA adducts + (S,G)	DNA adducts + (S,G)
Recovery 3 days	Cyp1A+ (G)	Cyp1A+ (S) Cyp1A+ (G)	MN+ (S) Cyp1A+ (S) Cyp1A+ (G)	MN+ (G) GST + (S) GST + (G) Cyp1A+ (S) Cyp1A+ (G)
Recovery 14 days		GST + (S) Cyp1A+ (G)	MN+ (S) GST + (S) GST + (G) Cyp1A+ (G)	GST + (S) GST + (G) Cyp1A+ (S) Cyp1A+ (G)

1) See appendix 6.

5 Fitness reduction *vs* biomarker responses

In this chapter the main results from the early life stage tests performed in BioSea JIP (including the Norwegian Research Council's PROOF funded additions, see Introduction) are presented together with comments on the biomarker responses observed in the adult animals used in the same experiments.

5.1 Test performance

5.1.1 Molluscs

Early life stage tests with mussels usually cease after 2 days when the larvae reach the D-shell stage. The elongated study used in this project commenced with the spawning of parents and the fertilisation of the eggs followed by an extensive period observing the

progress of developing larvae over 21 days. A technical challenge to this process presented itself in the form of extraneous organisms arriving in the seawater supply, despite the 40 μm mesh at the inflow. It cannot be discounted that the presence of these organisms disrupted the experiment. However, this disturbance was evident in all test conditions. Simple solutions were taken to reduce the problem, but in the future it is recommended that the test is performed using fully filtered seawater.

Unfortunately, it was not possible to perform early-life stage tests on *Chlamys islandica* within the allocated budget and therefore it was not possible to verify the results found with *Mytilus edulis.*

5.1.2 Shrimps

Early life stage tests on shrimp larvae worked well in both exposures, but especially in the Goliat exposure due to some improvements in the experimental setup. Shrimp larvae proved to be easy to work with in the lab, and can be strongly recommended for other early life stage test (for details see the report of Larsen, 2004). Generally, the two exposures correlate very well with each other, and it is clear that the larvae of Northern shrimp provide a useful crustacean model for studying effects on early life stages.

5.1.3 Sea urchins

Adult sea urchins were exposed for 7 months to Statfjord B oil in order to study effects on fitness in the next generation. A similar experimental design as that used for mussels was planned. Early life stage tests have been performed, but for reasons discussed in the echinoderm report these were not successful. Experience gained from the study suggests that using sea urchins for parental exposure experiments is not to be recommended due to problems with completion of gametogenesis in the laboratory. However, the process of carrying out a variety of early life stage experiments with this organism has improved our knowledge of best practice when studying sea urchins in the laboratory. It is recommend that for future experiments sea urchins are obtained from further north (e.g. the Tromsø area), that they are collected earlier in the season (January-February), and that even greater care is taken to maintain them in the laboratory at the temperature, light, and feeding conditions they experience in the field. Improved filtration of the inlet water serving the new larvae exposure system is required to avoid unwanted introduced organisms interfering with the normal development of the test larvae.

5.2 Reduced fitness for shrimps exposed to oil

Shrimp embryos were exposed for 3 months to oil and transferred to clean water after hatching. Exposure of larvae after hatching was also tested on control larvae and on larvae exposed to 90 µg/l THC as embryos. Table 12 and 13 give the main results from statistical comparisons of fitness data from oil exposed shrimps.

5.2.1 Mortality of adult shrimps

- The high concentration group of adult shrimps in the Statfjord experiment showed dramatically increased mortality after 3 months exposure.

5.2.2 Mortality of larvae

- The results on larvae revealed a dose dependent increase in mortality of larvae exposed during embryonic development, in both studies.

- Increased mortality was observed for larvae exposed to 21 and 90 µg/l Statfjord oil as embryos, but the difference between control and exposed larvae was only statistically significant at the highest exposure for stage 3 larvae. In the Statfjord experiment larvae exposed to the lowest oil concentration as embryos seemed not to be affected.

- The percentage of larvae which died within 24 hours, and the percentage dying before they reached stage 2, showed a clear dose dependency in the Goliat exposure. Significantly higher mortality was observed for shrimp larvae exposed to 5, 26, 112 µg/l Goliat oil than for control larvae.

- Exposure of shrimp embryo to Statfjord oil was more severe than exposure of the larvae. Mortality of larvae was higher for shrimps exposed to 90 µg/l THC as embryos than for shrimp larvae exposed only after hatching. Shrimps exposed both as embryo and larvae did not experience higher mortality than those exposed only at the embryo stage.

- There was some indication in the Statfjord study that mortality increased at the time of moult. This was not the case in the Goliat study.

5.2.3 Development time

Only small differences in development time were observed:

- Tendency to shorter development time from stage 1 to stage 2 larvae in the Statfjord oil exposure shrimp compared against controls, but a tendency to a longer development time from stage 2 to stage 3 indicated by an extended inter-moult period (stage 2-3) for larvae exposed to 4, 21 and 90 µg/l Statfjord oil as embryos.
- Tendency to longer development time in embryo exposed to 5 and 26 µg/l Goliat oil and significantly longer development time for larvae exposed to 112 µg/l. Tendency to longer development time from stage 2 to 3 for embryo exposed to 112 µg/l Goliat oil.

5.2.4 Fitness reduction *vs* biomarker response for shrimps

In the bottom part of tables 12 and 13 significant biomarker responses observed in adult shrimps have been included to compare at what concentrations and at what time fitness is reduced and biomarkers give a signal. Lysosomal membrane stability (NRRT), alkaline unwinding (AU), GST, Catalase (CAT) and TOSC all gave significant biomarker signals in shrimps.

In the Statfjord experiment significantly increased mortality was observed for shrimp larvae exposed for 3 months to 90 µg/l THC as embryos, and a tendency to increased mortality and changed development time was observed at lower concentrations (table 12). One month exposure to 4 and 21 µg/l oil did not induce significant biomarker signals in adult shrimps, but significant responses were observed for shrimps exposed to 90 µg/l (NRRT, AU, TOSC). Significant biomarker responses were observed in shrimps exposed to all the tested concentrations of oil (4, 21 and 90 µg/l) following 3-5 months exposure. We can conclude that NRRT, AU, CAT, GST, and TOSC gave significant responses at 4 µg/l and/or 21 µg/l following 3-5 months exposure, and that statistically significant reduction of fitness was not observed at these concentrations, although there were tendencies to increased mortality and changed development time.

In the Goliat experiment only NRRT was analysed both after 1 and 3.5 months exposure, the other biomarkers were only analysed following 1 month exposure (table 13). Increased mortality of larvae was observed for shrimp larvae exposed to 5, 26 and 112 µg/l oil as embryos, and significant effect on development time was detected at the

highest concentration. Significantly reduced fitness of shrimp larvae and significant biomarker responses were observed for shrimps exposed to 5, 26 and 112 µg/l Goliat oil. The biomarker responses were observed following 1 month exposure (NRRT also following 3.5 months exposure), and the fitness reduction following 3 months exposure to oil. The biomarker responses may occur earlier in time than following 1 month exposure, but this was not measured. Likewise, the effects on fitness of shrimp larvae may also have occurred after a shorter exposure (e.g 1 month) which was not tested either. Therefore, interpretations of the biomarker responses and effects as function of exposure times should be made cautiously.

Table 12. Fitness data from the **Statfjord B** exposure of shrimps (Larsen, 2004). Statistical comparison of data from the early life stage tests on **shrimp larvae**. Shrimp embryos were exposed to Statfjord B oil for 3 months, and then kept in clean water after hatching. Two additional treatments were included: control embryos exposed to 90 µg/l oil after hatching, and embryo *and* larvae exposed to 90 µg/l oil.

+++ Significant difference between control larvae and larvae from exposed parents/exposed larvae ($p<0.05$)
- : tendency to shorter development time for exposed larvae
+ : tendency to increased mortality/longer development time for exposed larvae
n.s.: No statistically significant difference from the control level ($p<0.05$)

Parameter (fig no. in Crustacean report):	**Larvae in clean water, embryo exposed to:**			Control embryo exposed to 90 µg/L THC after hatching	Embryo and larvae exposed to 90 µg/L THC
	4 µg/L THC	**21 µg/L THC**	**90 µg/L THC**		
Mortality					
Accumulated mortality 1st day of 100% stage 2 (fig 42a)	n.s.	+ n.s.	+ n.s.	+ n.s.	+ n.s.
Accumulated mortality 1st day of 97-100% stage 3 (fig 42b)	n.s.	+ n.s.	+++	+ n.s.	+++
Development time					
Development time; days to 50% stage 2 (fig 48)	- n.s.	- n.s.	- n.s.	- n.s.	- n.s.
Development time; days to 50% stage 3 (fig 49)	n.s.	n.s.	+ n.s.	n.s.	n.s.
Days in inter-moult (stage 2-3) (fig 50)	+ n.s.	+ n.s.	+ n.s.	n.s.	n.s.
Biomarker responses in adult shrimps					
1 month exposure					
NRRT	n.s.	n.s.	p<0.001		
AU	n.s.	n.s.	+++		
CAT	n.s.	n.s.	n.s.		
GST	n.s.	n.s.	n.s.		
TOSC ($ROO^{\cdot}$)	n.s.	n.s.	- - -		
3-5 months exposure					
NRRT	n.s.	p<0.001	p<0.0001		
AU	+++	+++	+++		
CAT	n.s.	+++	n.s.		
GST	- - -	- - -	- - -		
TOSC ($ROO^{\cdot}$)	- - -	- n.s.	- - -		

- - - Significantly reduced activity/capacity of biomarker
Significantly more DNA strand breaks (AU), lower membrane stability (NRRT), higher enzyme activity (CAT)

Table 13. Fitness data from the **Goliat** exposure of shrimps (Larsen, 2004). Statistical comparison of data from the early life stage tests on **shrimp larvae**. Shrimp embryos were exposed to Goliat oil for 3 months, and then kept in clean water after hatching.

p = significance level: Significantly higher mortality/longer development time for exposed embryos than control larvae
+ : tendency to increased mortality/longer development time for exposed embryos
n.s.: No statistically significant difference from the control level (p<0.05)

Parameter (fig no. in Crustacean report):	**Larvae in clean water, embryo exposed to:**		
	5 µg/L THC	**26 µg/L THC**	**112 µg/L THC**
Number of larvae hatched pr female (fig 51)	**n.s.**	**n.s.**	**n.s.**
Mortality			
Percent dead larvae of total per female (fig 52a)	p<0.0001	p<0.0001	p<0.0001
Percent dead larvae including embryos of total per female (fig 52b)	p<0.001	p<0.0001	p<0.0001
Accumulated mortality 1st day of 100% stage 2 (fig 54a)	p=0.022	p=0.01	p=0.001
Accumulated mortality 1st day of 97-100% stage 3 (fig 54b)	+ **n.s.**	+ p=0.064	p=0.001
Percent dying before stage 2 relative to total mortality (fig 55)	p=0.058	p=0.017	p=0.01
Development time			
Development time; days to 50% stage 2 (fig 60a)	+ p=0.2	+ p=0.153	p=0.013
Development time; days to 50% stage 3 (fig 60b)	**n.s.**	**n.s.**	+ p=0.17
Days in inter-moult (stage 2-3) (fig 61)	**n.s.**	**n.s.**	**n.s.**
Biomarker responses in adult shrimps			
1 month exposure			
NRRT	**n.s.**	p<0.0001	p<0.0001
AU	**n.s.**	**n.s.**	+++
CAT	**n.s.**	**n.s.**	- - -
GST	**n.s.**	**n.s.**	**n.s.**
TOSC ($OH^{\cdot}$)	+++	+	+++
3.5 months exposure			
NRRT	p = 0.014	p<0.001	p<0.0001

- - - Significantly reduced activity (CAT)
Significantly more DNA strand breaks (AU), lower membrane stability (NRRT), higher TOSC

5.3 Reduced fitness for mussels exposed to oil

Hypothesis : If parental exposure to oil affected the fitness of the larvae, we would expect higher mortality, a higher percentage of deformed larvae, slower development and hence a smaller size of larvae from oil exposed mussels than control mussels. This may be followed by a lesser chance of survival to metarmophosis. At lower exposure concentrations we can expect effects later in larval development than at higher concentrations and possibly a different mechanism of effect. Exposing the larvae directly to oil may give even stronger impairments.

Experimental consideration : Some of the variation may be explained by non-optimal conditions and we cannot completely exclude that some experimental differences (flow, feeding) existed between the different replicates. Nevertheless, they were minimized and we tried to give all groups similar exposure conditions. In the mussel early life stage, 4 replicate 1L-bottles were used for each treatment including the control. As the "maternal transfer" larvae and the direct oil exposure of the larvae were housed in two different locations a separate control group was included for each set of conditions. Approximately 20 fertilized eggs per ml was added to each bottle at the onset of the experiment. Sub-samples were taken for analysis from each test chamber 2, 7, 14 and 21 days after the transfer. The developmental stage and percentage normal and deformed larvae were recorded. Usually 200 larvae per treatment were classified at each sampling time (see table 14 where the number of larvae studied from each treatment at each sampling time is given). The size (rate of growth) of larvae was evaluated using 20 larvae per replicate (80 for one group). Our experimental setup was not directed at estimating a direct measurement of mortality. It is however possible to get indications of short-term mortality and the chance of survival by examining the record of % eggs and trochophores still present after 2 days in the exposed chamber compared to the control chambers. Trochophores still present after 2 days have a reduced chance of surviving to become larvae. In normal conditions, *Mytilus edulis* embryos will reach the first larval stage called "D-shell" after 48 h at 15°C (Strathmann, 1987; His et al., 2000). A significant shift in this time is indicating anomalies in embryonic development. Two scenarios may follow:

i) in the worst case, the normal development of the embryos is completely inhibited or even so affected that the short term chance for survival is extremely reduced i.e. trochophores will die within the following days of

development or will not give a viable D-shell. The energy storage transferred from male and female gametes is limited. Hence, larvae exhibiting significant delay in development during the embryonic stage will risk exhaustion of its reserve as it can not feed itself. This is only when the larvae have completed the D-shell with a fully developed feeding organs (velum) and digestive system that they can feed on algae;

ii) ii) the trochophores develop but at a slower rate. Even though this delay does not directly impair the development of the larvae to metamorphosis, it exposes them to stronger environmental stress and increase chance of loss through for example dispersion, predation and disease.

For some literature discussion on developmental effects from different exposure, please see for example Nice et al., 2000; Wedderburn et al., 2000; Spangenberg and Cherr, 1996; Hoare et al., 1995; Widdows, 1991.

Summary of results: Table 14 gives the main results from statistical comparisons of fitness data from oil exposed mussels.

5.3.1 Seven months parental exposure to 3, 15, 63 µg/L oil

The larvae were kept in clean water (from fertilisation until day 21).

- Day 2 – Altered growth for the larvae from mussels exposed to 3 and 63 µg/l oil. Additionally, in the 63 µg/l group, the average record of 52 % eggs and trochophores (but only 13% in the control) indicates slow and impaired development, and probably relatively high short-term mortality.

- Day 21 – Reduced growth in the larvae from mussels exposed to 3 and 15 µg/l oil indicated by less larvae at the umbo stage compared to the control group. No difference between the controls and 63 µg/l groups.

The results observed in the 63 µg/l group indicate a combination of reduced survivorship and possibly hormetic compensation of the survivors in an attempt to regain homeostatic balance. A large number of larvae are dying during the early developmental stage (day2) but the survivors grow faster (day 21). The development of the larvae from parents exposed for 7 months at the two lowest concentrations is normal within the first two days. However, some disturbances inherited from their parents may exist, as indicated by the slower rate of growth in the 3 µg/L group which also has 15% of slight deformation (3% in control). Hormetic responses, indicated by a faster growth

at day 7, may have an energetic cost and this resulted in slower growth in the long-term as less larvae reached the umbo stage later in the development (at day 21).

5.3.2 Direct exposure to 63 µg/L oil

- The percent fertilized eggs was 6 % lower for directly exposed eggs and sperm (from control parents) than for eggs in clean water. The difference was not statistically significant.
- Day 2 -Significantly more deformed larvae after 2 days exposure (19%) than in control (4%) and significantly smaller larvae after 2 days exposure.

In this group, there is no indication of short-term mortality of the larvae as the record of eggs and trochophores is not different than in the control. However, there are significantly less normal larvae and this indicates that a large percentage of the larvae will have a reduced chance to develop normally in the latter stages.

5.3.3 Parental and direct exposure to 63 µg/L oil

- The fecundity success was 34 % lower for this group than for the control ($p<0.05$).
- Significantly smaller larvae after 2 days exposure.
- Significantly slower development, and very likely high mortality indicated by the observation of 84% eggs and trochophores (but only 5% in the control) and only 7% of the larvae at the normal D-shell stage after 2 days.

In view of the low % of fecundity success, the presence of a large number of trochophores at day 2 (when the larvae should be at the D-shell stage) and very few D-shells, this group is obviously performing badly and the mortality is probably high within the first days of development (from the blastula to the D-shell). It is likely that many embryos (trochophores) died before they even reached the D-shell stage. However, the relatively few surviving larvae showed a faster veliger growth during later life stages, a feature which we attribute to a hormetic response. As there is larger effect in this group than the previous (larvae exposed but control parents), we have to conclude that maternal transfer is additively impairing the development of these offspring. That resulted in relative short-term mortality (as expressed by the relatively

large % of trochophores still present at day2) and hormetic growth compensation of the survivors in later life stages (no difference at day 21).

Hormesis : Hormesis usually refers to the stimulation of organism performance occurring at low levels of exposure to agents that are harmful or toxic at high levels of exposure. Such responses can also give important early warning signals of disturbances as it appears to be beneficial in the short-term (better growth at day 7 in the maternal transfer group at the lowest concentrations) but can indicate reduced fitness on the long term (reduced growth at day 21). Stimulation of growth, reproduction and other relevant parameters is also a well-known phenomenon (for relevant references see for example Valery Forbes (2000) or a number of papers by Calabrese et al. and Stebbing et al.[2]). In the experiment with mussel larvae there was an indication that such a phenomenon occurred. A possible explanation is that modifications of the veliger physiology are triggered by oil compounds or inherited from maternal transfer. As a consequence the surviving larvae would accumulate lower reserves of lipid, and in an attempt to regain homeostasis, maintain a rapid growth rate which ultimately is likely to reduce metamorphosis success rate. Some compensatory effects in the development time between stage 1 to 2 and stage 3 in the shrimp larvae experiment were also indicated. They may also be attributed to hormesis.

Conclunding remark : The mussel bioassay is usually stopped after 2 days as the records obtained after this time may be sufficiently predictive of the survival chances of the larvae during the latter stages of development. However, the continuation of the test is useful as changes may occur when the larvae start to feed and some compensatory effects may appear. A combination of reduced survivorship and physiological changes consecutive to parental exposure and direct exposure and hormetic effects for the survivors was indicated. The larval development in all scenario groups was affected.

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[2] In the program for the next SETAC 2005 meeting in Lille (France), there is a separate session for low dose effects, including hormesis.

5.3.4 Fitness reduction *vs* biomarker response for mussels

In the bottom part of table 14 significant biomarker responses observed in the adult mussels have been included to compare at what concentrations and at what time fitness is reduced and biomarkers give a signal. Only Comet assay and GST gave significant biomarker signals for adult mussels in this experiment. Comparing all cells from exposed animals to control cells there were significantly more DNA strand breaks in haemocytes sampled from all 3 groups of oil exposed mussels following both 1 month and 7 months exposure. Comparison of mean DNA damage in individual mussels show that less response was observed following 7 months than 1 month exposure, especially in the highest exposure concentration. Significantly higher GST activity was detected following 1 month exposure to 63 µg/l, but no significant responses were detected following 7 months. In the next chapter we try to explain such non-linear dose (and time) relationships. We can conclude that the comet assay may be able to provide an early warning of reduced fitness in mussels. The comet assay responds in the same range of concentrations that reduces the fitness of the next generation, but indicates detriment following only 1 month exposure. The fitness reduction was observed following 7 months exposure. We do not know if fitness had been reduced following only one month exposures of the adults.

Table 14. Statistical comparison of data from the early life stage tests on mussel larvae. The parent mussels were exposed to Statfjord B oil for 7 months, and the embryo and larvae were kept in clean water. Two additional treatments were included: control embryo/larvae exposed to 63 µg/l oil starting at fertilization, and both parent mussels and embryo and larvae were exposed to 63 µg/l oil.

Significant difference between control larvae and larvae from exposed parents/exposed larvae ($p<0.05$)

	Control	Larvae in clean water Parent mussels exposed to:			Control larvae[1] exposed to 63 µg/L THC	Parents and larvae exposed to 63 µg/L THC
		3 µg/L THC	15 µg/L THC	63 µg/L THC		
Fecundity success (1.5 h)	94 % n = 125	98 % n = 451	95 % n = 544	94 % n = 225	88 % n = 288	60 % n = 179
2 days exposure:						
% D-shell larvae	84 % n = 525	82 % n = 343	90 % n = 430	40 % n = 254	58 % n = 249	7 % n = 70
% deformed D-shell larvae	2.5 %	15 %	6.5 %	9 %	19 %	10 %
Length of larvae	108 µm n = 160	105 µm n = 80		102 µm n = 80	98 µm n = 67	91 µm n = 10 2)
7 days exposure:						
% normal veliger larvae	63 % n = 700	82 % n = 405	80 % n = 459	67 % n = 643	69 % n = 472	29 % n = 152
% deformed larvae	30 %	19 %	17 %	30 %	36 %	53 %
Length of larvae	115 µm n = 160	121 µm n = 78	114 µm n = 80	113 µm n = 80	107 µm n = 76	104 µm n = 63
21 days exposure:						
% umbo larvae[4]	39 % n = 192	18 % n = 183	34 % n = 728	41 % n = 113	No data	59 % n = 169
% deformed larvae	2%	2%	3%	7%		10%
Length of larvae	144 µm n = 100	130 µm n = 80	136 µm n = 80	142 µm n = 60		152 µm[3] n = 20
Biomarkers in adult mussels [5]						
1 month exposure						
Comet		p < 0.05	p < 0.05	p < 0.05		
GST		**n.s.**	**n.s.**	p < 0.05		
7 months exposure:						
Comet [6]		p < 0.05	p < 0.05	p < 0.05		
GST		**n.s.**	**n.s.**	**n.s.**		

1) Fertilisation and first 21 days of development in 63 µg/l THC
2) Few larvae had developed to the D-shall stage – few larvae to measure.
3) Larger larvae than in control most likely because the small larvae died before day 21, only the larvae that grow fast survive.
4) Too few replicates for statistical analysis.
5) Only Comet assay and GST gave statistically significant biomarker signals in adult mussels.
6) Less difference between control and exposed after 7 months than 1 month, especially in 63 µ/l oil.

5.4 Fitness reduction *vs* biomarker responses in fish

Fitness was not studied in the BioSea JIP experiments where cod were exposed to Statfjord and Goliat oil. Lower concentrations of oil and longer exposure times have been tested in the fitness experiments with invertebrates in BioSea than in fish reproduction tests performed at Akvamiljø in previous projects, hence it is difficult to compare the results we have on fitness reduction for invertebrates and fish. If we ignore the shorter exposure time for fish, the biomarkers appear to give an earlier warning for fish than for the invertebrates. But we can not exclude the possibility that fitness may be reduced also for fish early life stages following exposure of parent fish for 7 months.

Fish have been exposed for 5-6 weeks to oil in previous experiments (Bechmann *et al*. 2004, SPE paper). These are short term exposures compared to the invertebrate exposures in BioSea (3 months for shrimp embryos and 7 months for adult sea urchins and mussels). Reduced fitness of fish larvae due to parental and larvae exposure to 100 µg/L was observed in one experiment, and parental exposure to 700 µg/L caused reduced fitness of larvae in another experiment. It is the last experiment that is most comparable to BioSea because the CFS was used for mixing oil and sea water (in the first fish exposure ultrasound was used). Either fish are better at detoxification/repair than invertebrates, or short exposure times for parent fish cause less effects than longer exposures. Fitness parameters for fish have not been studied at RF-Akvamiljø at lower concentrations of oil than 100 µg/L. It would be possible to carry out long term exposures of fish to lower concentrations (~ 5 µg/L) to study possible biomarker responses at similar conditions as those used in the mussel BioSea exposure.

6 Interpretation of biomarker signals

Accurately interpreting biomarker signals requires a sound basis of biological knowledge on the species under investigation. Each biomarker has a ‘window’ in concentration and time where it responds. The response may be linear or non-linear. Some biomarkers are responsive for a large range of concentrations, others respond only in a relatively narrow concentration range. Hence a lack of response does not necessarily mean there has been no exposure or effect on fitness.

In this chapter theoretical dose- response curves for biomarkers relative to fitness reduction for representative species will be presented and explained. One purpose is to provide a conceptual framework that the use of biomarkers for early warning of fitness effects can be understood within. Furthermore, these theoretical curves can be used for making testable hypothesis, and they can be useful when designing experiments for future projects where the aim is to learn more about biomarkers as early warnings of reduced fitness. It is recommended that the considerations given here will be adopted and developed further in the "Interpretation Handbook" project activities in 2005 (RF-Akvamiljø for Total E&P Norge AS).

In figure 3 three biomarkers with linear dose-response relationships are presented. Biomarker 1 responds at a lower exposure concentration than biomarker 2, which responds a lower concentration than biomarker 3, but the biomarker signals increase continuously with increasing exposure concentration up to the highest (relevant) concentrations. PAH metabolites in bile of fish and DNA adducts are biomarkers with response curves similar to these. Biomarker 1, 2, 3 may be measured in a model species (e.g. mussel or cod) caged at different distances from the discharge. Species A, B and C in the bottom panel of figure 3 represents the most sensitive species in the ecosystem (A), the 'medium' sensitive species (B), and the species most resistant to the discharge (C) (see figure 4). These three representative species in the ecosystem experience reduced fitness at different exposure concentrations. An increased response of biomarker 1 is an early warning for reduced fitness of all the three representative species (A, B and C). An increased response of biomarker 2 is an early warning for reduced fitness of species B and C, indicating that only the most sensitive species experience reduced fitness at this concentration. An increased response of biomarker 3 is only an early warning for reduced fitness of the toughest species (represented by species C). A low biomarker signal indicate an early warning, but as the biomarker signal increases it gives a later warning relative to the concentration where reduced fitness is observed.

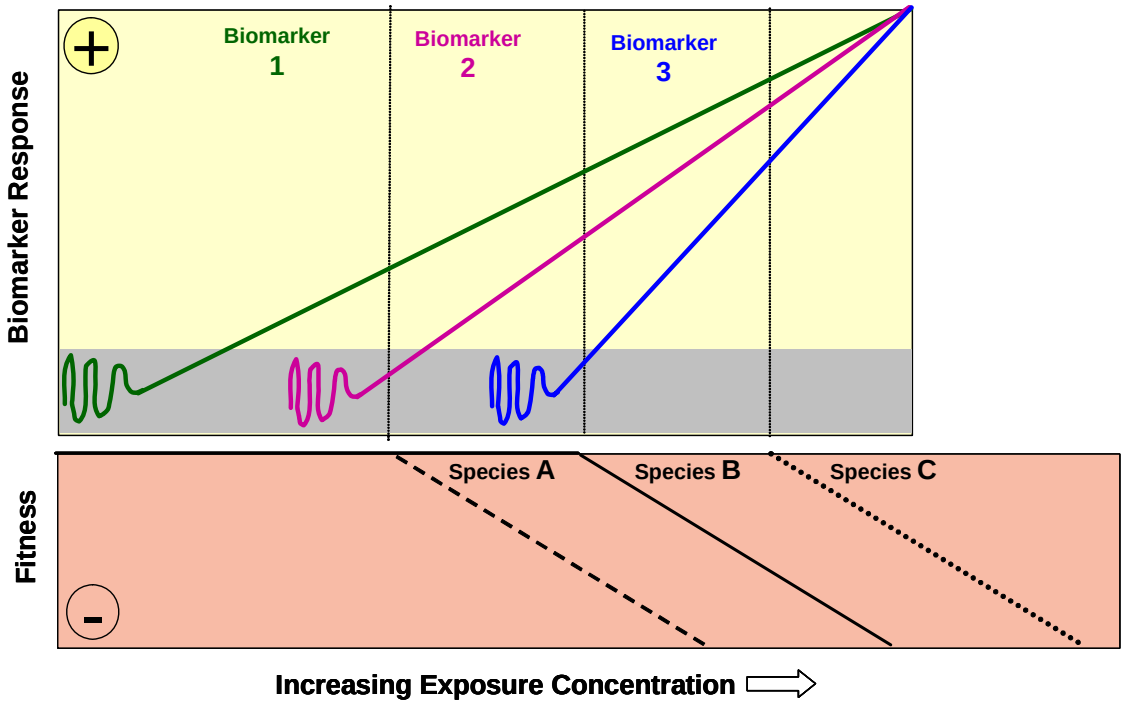

Figure 3. In the **top panel** three biomarkers with linear dose-response relationships are presented. Biomarker 1 responds at a lower exposure concentration than biomarker 2, which responds a lower concentration than biomarker 3, but the biomarker signals increase continuously with increasing exposure concentration up to the highest (relevant) concentrations. The grey area indicates the natural variability of the biomarker response. The **bottom panel** shows reduced fitness at different exposure concentration for 3 representative species in the ecosystem. Species A is representing the most sensitive species, species B has medium sensitivity to the discharge, and species C is a tough species that only experience reduced fitness at very high exposure levels (see text for more details).

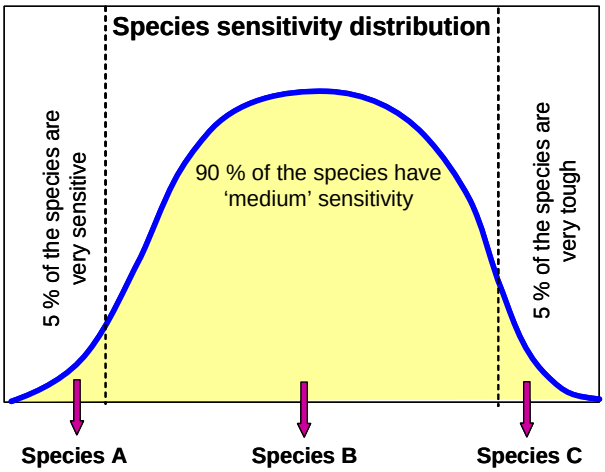

Figure 4. Species sensitivity distribution. Species A is representing the most sensitive species, species B has 'medium' sensitivity to the discharge, and species C is a tough species that only experience reduced fitness at very high exposure levels. These three representative species are used to illustrate theoretical correlations between biomarker responses and reduced fitness in figure 3 and 5.

In the top panel of figure 5 three biomarkers with non-linear dose-response relationships are presented. The three biomarkers have different sensitivity to the exposure. Each biomarker has a 'concentration window' where it responds. Biomarker 1 responds at a lower exposure concentration than biomarker 2, which responds a lower concentration than biomarker 3. At relatively low concentrations the biomarker signals in figure 5 increase with increasing exposure concentration before a plateau level is reached, and finally the signal is reduced to below the control level at higher concentrations. Non-linear dose-response relationships may be relevant especially for enzymatic biomarkers (e.g. EROD, Catalase, GST) and possibly for biomarkers that depend on enzymatic activity (e.g. induction of DNA repair enzymes may affect the level of DNA strand breaks). For other biomarkers reduced response compared to the control level is not possible/relevant, but even for such biomarkers a plateau level may be reached at high exposure concentrations. An increased response of biomarker 1 is an early warning for reduced fitness of all the three representative species (A, B and C). An increased response of biomarker 2 is an early warning for reduced fitness of species B and C indicating that only the most sensitive species experience reduced fitness at this concentration. An increased response of biomarker 3 is only an early warning for reduced fitness of the toughest species (represented by species C).

Biomarkers with non-linear dose-response curves are inhibited at higher concentrations. Biomarker 1 is reduced relative to the control level in a range of concentrations where reduced fitness is observed for the most sensitive species (represented by Species A). Biomarker 2 is reduced relative to control in a range of concentrations where reduced fitness is observed for most species, only the toughest species (represented by Species C) do not experience reduced fitness in this range of concentrations. Biomarker 3 is reduced relative to control in a range of concentrations where reduced fitness is observed for all the species.

The main challenge in the interpretation of results from biomarkers with non-linear dose-response curves is to be able to tell when no response means too low exposure to give a signal, and when it indicates too high exposure – close to inhibition of the biomarker signal. It is possible to interpret results from these types of biomarkers from laboratory studies which include frequent sampling and several exposure concentrations. With more detailed knowledge of the dose-response curves and possible time-related variation of response, these type of biomarkers can also be used in

animals caged at different distance from the discharge point. When wild caught animals are used for biomonitoring, biomarkers which respond only in a limited range of relevant concentrations and are time-dependent (like the biomarkers in figure 5) can only be used in addition to biomarkers with more linear responses and that respond in a wider range of concentrations and time (similar to the biomarkers shown in figure 5).

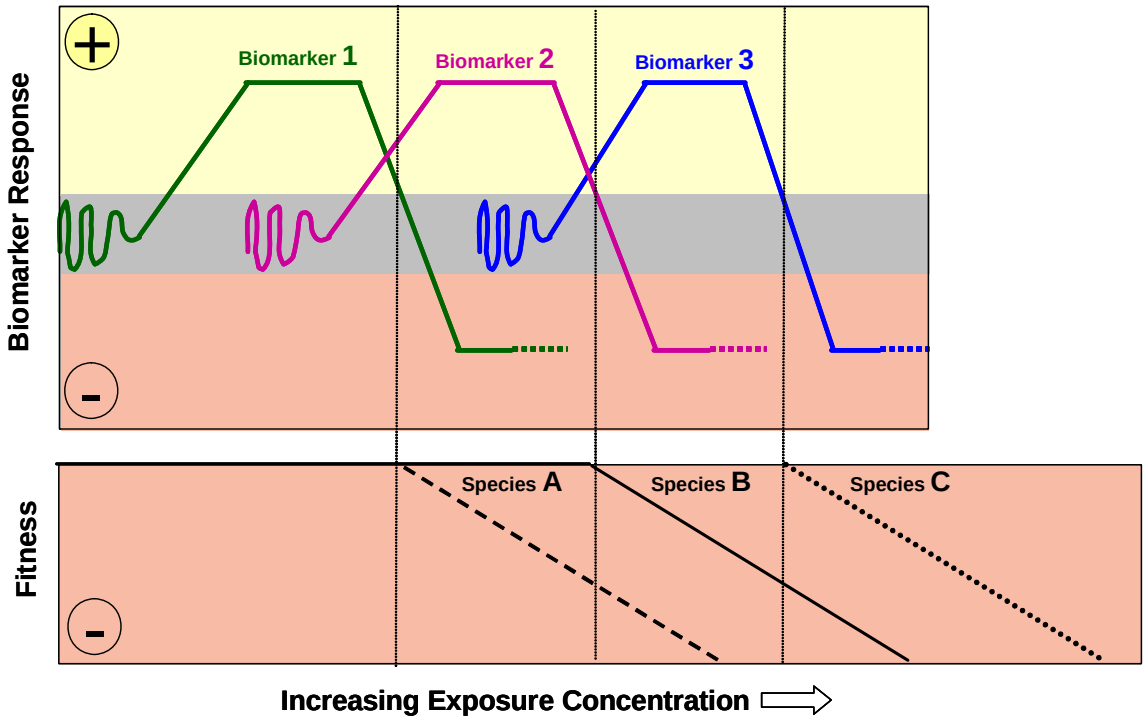

Figure 5. In the **top panel** three biomarkers with non-linear dose-response relationships are presented. Each biomarker responds within a limited range ('window') of concentrations. Biomarker 1 responds at a lower exposure concentration than biomarker 2, which responds a lower concentration than biomarker 3. The grey area indicates the natural variability of the biomarker response. The **bottom panel** shows reduced fitness at different exposure concentration for 3 representative species in the ecosystem. Species A is representing the most sensitive species, species B has medium sensitivity to the discharge, and species C is a tough species that only experience reduced fitness at very high exposure levels (see text for more details).

Some biomarker signals remain constant when the animal is continuously exposed to the same concentration, e.g. PAH metabolites in the bile of fish (Jonsson, 2004). Other biomarker signals, and effects on fitness, may not be constant with increasing exposure time. In figure 6 below, theoretical biomarker responses with increasing exposure time are illustrated. The type of time dependent biomarker responses described in figure 6 are relevant for enzymatic biomarkers (e.g. EROD, Catalase, GST) and possibly for biomarkers that are linked closely to the activity of enzymes affected by the exposure.

It is likely that the concentration of contaminants in the target tissues of the animal needs to reach a threshold level before the biomarker signal is induced (top panel of figure 6). This means that longer exposure time may be needed at low concentrations than at higher concentrations to get a biomarker signal. At medium concentration (mid panel, figure 6) a rapid increase in the biomarker signal occurs, but long term exposure causes inhibition of the response. At high exposure concentration only the reduced signal may be detected.

More data is needed to test whether the theoretical biomarker responses presented in figure 3, 5 and 7 are relevant for the range of concentrations and exposure times encountered in the sea.

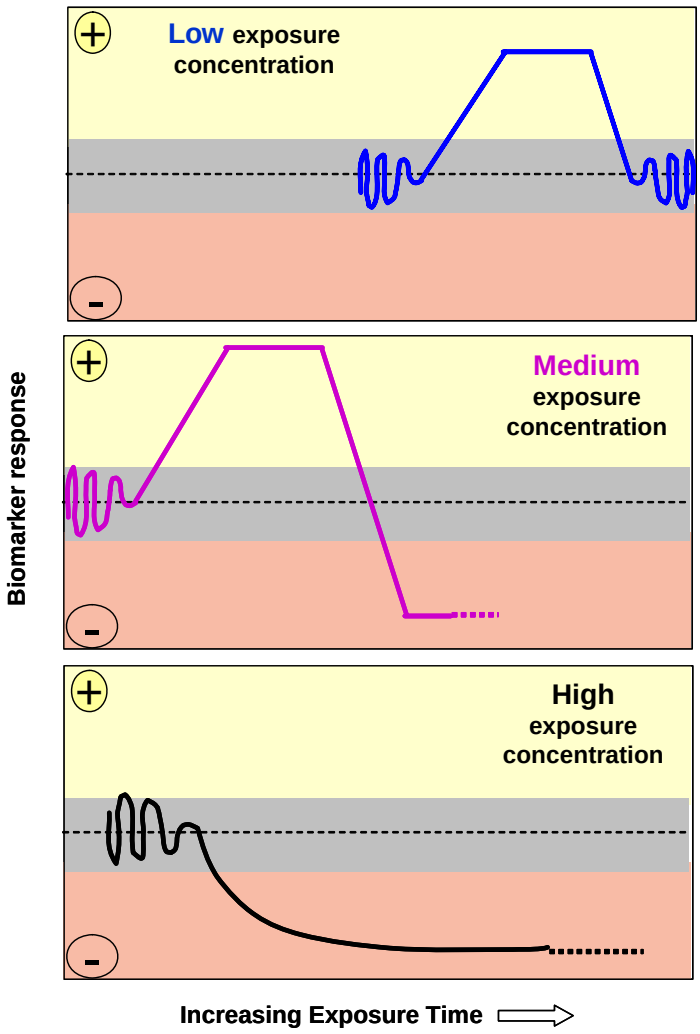

Figure 6. Examples of theoretical biomarker responses with increasing exposure time for animals exposed to low, medium and high exposure concentrations of a toxic compound.

7 Biomarker responses in cod sampled in the field and in the laboratory

In the report called “Biomarker background levels in Arctic marine species – Part II” Børseth & Sundt present background levels for biomarker responses in fish collected in Arctic waters (Børseth & Sundt, 2004). Some of these biomarkers have also been analyzed in the BioSea cod exposures (Skadsheim, 2005). In this chapter the background levels of PAH metabolites in bile, GST activity, DNA adducts, Vtg and Zrp in cod collected in Arctic waters are compared to the level in cod from the BioSea laboratory experiments.

The level of PAH metabolites in the bile measured by FF and GC-MS

Generally higher levels of PAH metabolites was observed in the bile of laboratory control fish (Barents Sea cod) than in field collected cod. Possible explanations are that the lab cod have been exposed to PAHs in the aquaculture facility (e.g. boat engines) before they were transported to Akvamiljø, or that the control fish have been contaminated in the lab. (There were, however, higher level of PAH metabolites in the bile of the oil exposed cods than in the control cod).

The level of PAH metabolites in the bile of wild caught fish from areas that may be contaminated must be compared to the level in fish caught in areas that is known to be clean, rather than to laboratory control levels. Likewise the level of PAH metabolites in the bile in caged fish must be compared to the level in fish from the same batch caged at clean site to avoid problems with the interpretation.

DNA adducts

The level of DNA adducts in the liver of Barents Sea cod and field collected cod was similar; 1 nmol/mol in field collected cod and 0.8 nmol/mol in the BioSea control cod (Barents Sea stock). The level of DNA adducts was higher in the Barents Sea cod exposed to 0.06 ppm oil (2.7 nmol/mol).

GST activity

Higher mean levels of GST activity was observed in Barents Sea and North Sea control cod from the BioSea exposures than the mean value from field collected cod (Børseth

and Sundt, 2004). Variable activity level in the BioSea experiments with time could be explained with the low number of replicates at each sampling time/treatment.

Difference in activity level between the BioSea exposures and the field collected cods may be explained by different sensitivity between different stocks, different season, sex differences, temperature, etc. To get better background levels in cod GST analyses have to be done on both sexes at different time of the year, and with more replicates like it was done in Børseth and Sundt).

The results from the BioSea JIP laboratory experiments indicate that GST activity in cod may not be a very sensitive biomarker for oil exposure. Only minor differences in activity between control and oil exposed groups of cod were observed, except at very high concentrations (1 mg/L oil + spike) (Skadsheim, 2005).

VTG and ZRP

The background level of Zrp and Vtg in cod collected in the field was similar to the values detected in juvenile Barents Sea and North Sea cods from the control and the different oil exposures in the BioSea experiments. Compared to cod exposed to 30 µg/L nonylphenol (reported in Børseth & Sundt, 2004) all Vtg and Zrp values detected in juvenile Barents Sea and North Sea cods from the control and the different oil exposures were low. In a previous experiment adult sheepshead minnows were exposed to 0.1 – 0.7 mg/L oil and Vtg and Zrp in males and females were measured. Generally there were small differences between control and exposed fish (both for males and females) compared to the large difference observed between male cods exposed to 30 µg/L nonylpehol and unexposed cod. There was, however a statistically significant increase in the mean level of Vtg in male fishes exposed to 0.4 and 0.7 mg/L oil compared to the control. There were no statistically significant changes in the plasma concentration of Zrp between different groups of oil exposed fish (females and males) (Bechmann *et al.*, unpublished manuscript from the DREAM validation fish project).

8 Research needs

In the following are some suggested follow-up activities to the reported results. (These are not by any means aimed at being exhaustive)

More knowledge on non-linear dose and time dependent responses

It is possible that the oxidative stress parameters (and other biomarkers) respond more rapidly following exposure to higher concentrations, and that the response had come and gone prior to the first sample period one month into the exposure (too long exposure). More knowledge about the shape of the dose-response relationships of biomarkers, and the changes in response profile with increasing exposure time, will increase the possibility of obtaining effective early warning signals when using biomarkers for biomonitoring. It may enable us to differentiate between early and late warnings, and help us decide whether something needs to be done with regard to the volume or composition of discharges, when the objective of monitoring is to ensure 'no harmful effects' occur. Experimental approaches necessary to further develop effective biomarkers include frequent sampling of caged organisms exposed in the field together with laboratory studies designed to better describe biomarker responses to chronic and pulsed exposures to low concentrations of pollutants (see below).

Impact of chronic low concentration exposures on fish fitness parameters

Test fitness of fish larvae following exposure of parent fish to similar conditions as adult mussels in the BioSea exposure experienced. Are fish less sensitive than invertebrates?

Short(er) term exposure of mussels to study fitness parameters

To be able to say with confidence whether a biomarker signal should be interpreted as a danger of reduced fitness in the next generation we need to carry out more relevant shorter term exposures of parent animals during gonad development and study fitness parameters of the subsequent larvae.

Reduce 'background noise' for biomarker responses

More research on the variability of biomarker responses due to season, temperature, age, sex, etc. in addition to optimization of selected biomarker methods should increase the likelihood to distinguish better an early warning signal from 'background noise'.

Establish Comet assay on frozen samples

Most laboratories carry out comet assays on fresh samples, but at least two also use the method on frozen material. At Akvamiljø we have completed a pilot experiment to

establish the method for freezing samples, but without success. If the Comet assay is to be successfully used for biomonitoring in the future it is important to establish a successful method for freezing samples. The most cost-efficient way to do this would be to invite an expert in this field to come to RF-Akvamiljø to pass on their experience. There is no guarantee, however, that the Comet assay will work sufficiently well on frozen tissues to successfully support biomonitoring procedures.

Caged shrimps

Pilot study to test whether it is possible to successfully cage shrimps for use in biomonitoring studies. To include evaluation of a selection of biomarkers.

Pulsed exposure

It is likely that feral organisms will drift or swim in and out of the produced water plume. This raises the question of the reliability of random samples taken from organisms caught within the plume. How long does the biomarker response last following exposure? We recommend a laboratory study where relevant test organisms undergo pulsed exposures to simulated PW to discover how intermittent exposures influence the pattern of biomarker responses, see figure 7.

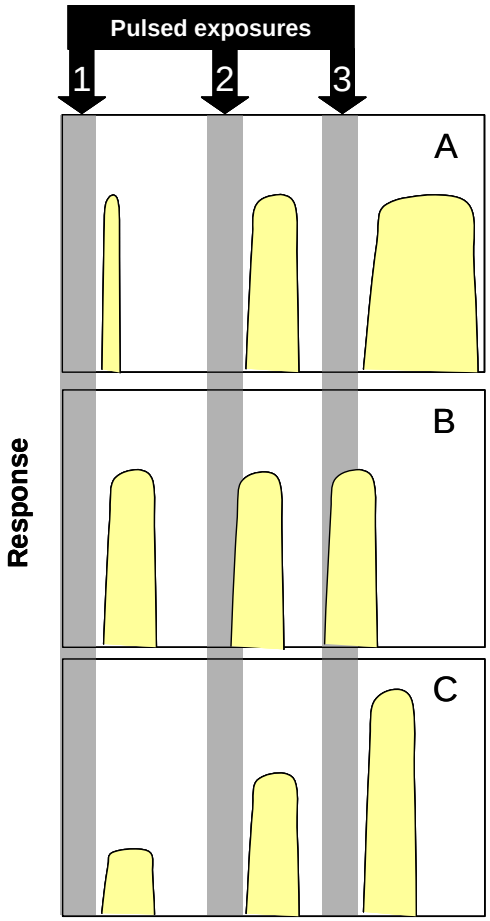

Figure 7. Pulsed exposure. In the field, animals are likely to be exposed repetitively to the discharge for relatively short periods each time. How will this type of exposure affect the biomarker response? This is an important question to answer to allow accurate interpretation of biomarker signals detected in biomonitoring studies. **A:** Will the biomarker response last longer when the animals have been exposed several times, than following the first exposure incident? **B**: Can we expect a more rapid biomarker response when the animals have been exposed several times, than that produced following the first exposure incident? **C**: Will the biomarker response increase in intensity when the animals have been exposed several times, than following the first exposure incidence? These are relevant questions when comparing responses in wild caught and caged animals.

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10 Appendix

Appendix 1. Relevant results from genotoxicity assays in other projects. +++ Significant increase in DNA damage compared to corresponding control level (p<0.05). **n.s.**: No statistically significant difference from the control level (p<0.05).

Species	Tissue	Exposure/Project info.	Genotoxicity test			
			Alkaline unwinding	Comet assay	Micronuclei	DNA adducts
Blue Mussel *Mytilus edulis*	Gill	Beep – Karmøy, Field val. PAH, Metals	**n.s.**	tendencies		
		Beep – Mosjøen, Field	**n.s.** tendencies			
		Beep – Sweden, field, harbour, industry	+++			
		Beep - Endocrine disrupters	+++	+++		
	Haemocytes	Rockness, oil spill, recovery		**n.s.**		
		Styrene (Ecopel)		+++		
Shore Crab *Carsinus maenas*	Hepato-pancreas	Beep - Karmøy, Field val. PAH, Metals	+++			
Spider Crab		Beep workshop 3, Endocrine disrupters	+++			
Corkwing wrasse *Crenilabrus melops*	Liver	Beep - Høgevarde, Bukkøy and Visnes, Field val. PAH, Metals	+++			
	Blood cells	Beep, Visnes, Field val. Metals		+++		
	Liver	Bleivik, Field val.; Oil spill (Aas report)	**n.s.**			
	Blood cells	Styrene (Ecopel)		+++		
Eelpout	Liver	Beep Sweden, Field val.:, harbour, industry	+++			
Barents Sea Cod *Gadus morhua*		Water Column Monitoring 2003. Field exposure (cages), Troll B Oil platform (500, 1000 m), 6 weeks			+++	**n.s.**
North Sea Cod *Gadus morhua*		CFS4 North Sea oil, **40 µg/L oil** (Aas paper)				+++
		Beep - Endocrine disrupters	+++			
Sheepshead minnow *Cyprinodon variegatus*		DREAM - A mixture of PAHs, **2 - 214 µg/L**				+++
		DREAM val. fish (Total), Statfjord B oil; **100-700 µg/L**				+++
Turbot *Scophthalmus maximus*		Beep - North Sea oil	+++			
	Gill	Beep - Endocrine disrupters	+++			

Appendix 2. Results from statistical analysis of the differences in **GST activity** between oil exposed and control animal in the BioSea JIP experiments.
+++ Significantly increased activity level
- - - Significantly reduced activity level
+ increase activity level, not significant
- Reduced activity level, but not significant
n.s. The difference between control and exposed was not statistically significant at the p<0.05 level.

Species Tissue	**µg/L THC (oil)**	**Exposure time**	**Low conc.**	**Medium conc.**	**High conc.**
Blue Mussel *Mytilus edulis* Hepatopancreas	Statfjord oil 3, 15, 6	1 month	+ **n.s.**	**n.s.**	+++
		7 months	- **n.s.**	- **n.s.**	+ **n.s.**
Arctic scallop *Chlamys islandica* Hepatopancreas	Goliat oil 2,14, 64	1 month	+++	+ **n.s.**	+ **n.s.**
Northern Shrimp *Pandalus borealis* Hepatopancreas	Statfjord oil 4, 21, 90	1 month	- **n.s.**	**n.s.**	+ **n.s.**
		5 (3) months	- - -	- - -	- - -
	Goliat oil 5, 23, 102	1 month	+ **n.s.**	+ **n.s.**	**n.s.**
Green Sea urchin *Strongylocentrotus droebachiensis* Gut	Statfjord oil 4, 29, 85	1 month	*Not analysed*		+ **n.s.**
		7 months	- **n.s.**	- **n.s.**	- **n.s.**
Atlantic cod *Gadus morhua* Liver	Statfjord oil 60, 250*	3 days	*Not tested*	**n.s.**	+++
		14 days		**n.s.**	**n.s.**
		24 days		**n.s.**	+ **n.s.**
Barent Sea Cod *Gadus morhua* Liver	Goliat oil 60, 250*	3 days		+++	
		17 days		**n.s.**	- **n.s.**
		31 days		**n.s.**	**n.s.**

* nominal conc. Corresponding to medium and high exposure for invertebrates (parallel exposures in same system), the low concentration was not tested on fish.

Appendix 3. Results from statistical analysis of the differences in **catalase activity** between oil exposed and control animal in the BioSea JIP experiments.
+++ Significantly increased activity level
- - - Significantly reduced activity level
+ increase activity level, not significant
- Reduced activity level, but not significant
n.s. the difference between control and exposed was not statistically significant at the p<0.05 level.

Species Tissue	µg/L THC (oil)	Exposure time	Oil concentration:		
			Low	Medium	High
Blue Mussel *Mytilus edulis* Hepatopancreas	Statfjord oil 3, 15, 6	1 month	+ n.s.	n.s.	+ n.s.
		7 months	n.s.	n.s.	+ n.s.
Arctic scallop *Chlamys islandica* Hepatopancreas	Goliat oil 2,14, 64	1 month	+++	+++	+++
Northern Shrimp *Pandalus borealis* Hepatopancreas	Statfjord oil 4, 21, 90	1 month	+ n.s.	+ n.s.	+ n.s.
		5 (3) months	+ n.s.	+++	+ n.s.
	Goliat oil 5, 23, 102	1 month	n.s.	n.s.	- - -
Green Sea urchin *Strongylocentrotus droebachiensis* Gut	Statfjord oil 4, 29, 85	1 month	Too low activity		
		7 months	+ n.s.	+++	+ n.s.

Appendix 4. Results from statistical analysis of the differences in **TOSC level** between oil exposed and control animal in the BioSea JIP experiments.
+++ Significantly increased level, - - - Significantly reduced level, + increase level, not significant, - Reduced level, but not significant. **n.s.** the difference between control and exposed was not statistically significant at the p<0.05 level.

Species Tissue	µg/L THC (oil)	Exposure time	Oil concentration:		
			Low	Medium	High
Blue Mussel *Mytilus edulis* Hepatopancreas	Statfjord oil 3, 15, 6	1 month	+ n.s.	+ n.s.	+ n.s.
		7 months	+ n.s.	- n.s.	+ n.s.
Arctic scallop *Chlamys islandica* Hepatopancreas	Goliat oil 2,14, 64	1 month	+++	+++	+ n.s.
Northern Shrimp *Pandalus borealis* Hepatopancreas	Statfjord oil 4, 21, 90	1 month	- n.s.	+ n.s.	- - -
		5 (3) months	- - -	-	- - -
	Goliat oil 5, 23, 102	1 month	+ n.s.	+ n.s.	n.s.

Appendix 5. List of biomarkers in relevant biomonitoring studies

BECPELAG

ICES biological effects monitoring in pelagic ecosystems workshop (BECPELAG). The aim was to establish suitable techniques for monitoring the effects of contaminants on pelagic ecosystems. Caged and field-collected animals. German Bight and four sites in a downstream transect from the Statfjord area.
Information from: Utvik. Produced water – Zero Discharge. Myth or reality? Presented at the Tekna meeting 15-16 January 2004, Stavanger, Norway.

Biomarkers in field-collected fish:

- fish larvae aberration (no response)
- EROD (cytochrome P4501A activity) (no response)
- Concentration of cytochrome P4501A (only response in German bight)
- Histopathology of fish larvae
- Histopathology of fish liver (significant effects on herring and saithe tissue integrity were found close to the Statfjord B platform + German Bight)
- DNA adducts in fish larvae (adducts in German Bight, but no difference between stations)
- VTG (no response)
- PAH metabolite level in bile (no response)

Biomarkers in caged Atlantic cod and Mussels

- Histopathological changes in hepatopancreas (basophilic cell volume) (mussel)
- Histochemistry (mussels)
- Lysosomal stability (effect close to the oil rig) (mussel)
- Acetyl cholin esterase (AChE) – cod and mussels in German Bight – response
- Benzo(a)pyrene hydroxylase (BaPH) was induced in mussels kept in inner German Bight
- Scope for growth (mussel) – no response
- Metallothionein in mussels – no response
- proteomics (mussels) – indications of response
- PAH metabolites – obvious gradients in caged cod
- EROD activity – no response
- Glutatione S-transferase (GST) activity increased at the sites closest to the oil rig
- DNA adducts in cod
- Vitellogenin level (VTG)

The Tampen study

Klungsøyr, J., Balk, L., Berntssen, M.H.G., Beyer, J. Melbye, A.G., Hylland, K. Contamination of fish in the North Sea by the offshore oil and gas industry. NFR project no. 152231/720. Summary report to NFR .
The Tampen/Statfjord area; the same as in BECPELAG, Sleipner and Egersundbanken (reference), collection of feral fish, no caging and no invertebrates.

- PAH metabolites in bile: FF and GC/MS (no response – like in feral fish in BECPELAG, but response in caged cod)
- Vitellogenin
- EROD
- Glutahion reductase
- Gluthation S-transferanse

- EROD (cyt P4501A activity)
- Liver somatic index
- Lipid peroxidation (TBARS)
- alpha-tocopherol (vitamin-E) (reduced vitamin E level in muscle and reduced SeGSH-Px indicate a using of anti-oxidants and onset of lipid radical scavenger enzymes)
- catalase
- Selenium dependent glutathione peroxidase activity (SeGSH-Px)
- Composition of fatty acids (and ratio of n3/n6 fatty acid)
- DNA adducts

The Troll study

Børseth & Tollefsen (2004). Water column monitoring 2003 (Report RF-2004/039).
The monitoring program for 2003 was performed in the area around the Troll B platform, and was based on the experiences and recommendations from BECPELAG. Caged Atlantic cod and blue mussels.

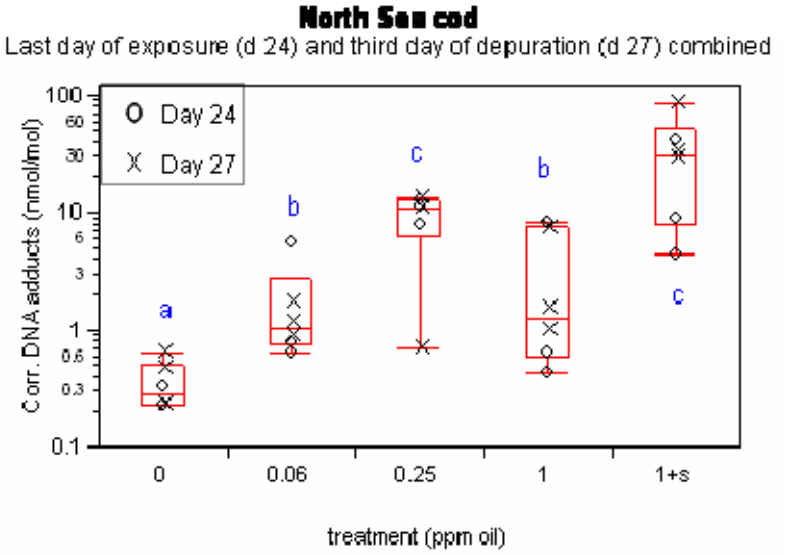
Cod biomarkers:

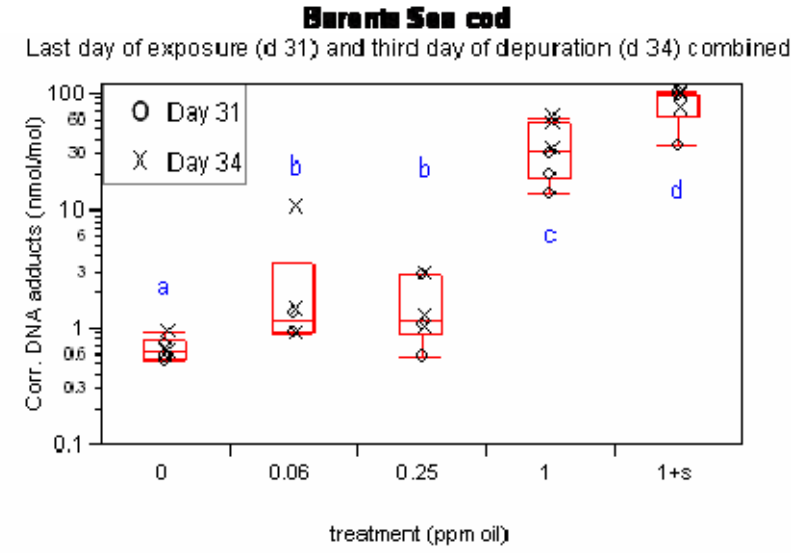
- Vitellogenin (VTG)
- PAH metabolites
- EROD activity
- GST activity
- DNA adducts
- Micronuclei assay
- Histochemistry
- Histopathology

Mussel biomarkers:

- BaPH activity
- Lysosomal response
- Histochemistry
- Histopathology

Appendix 6. Results from statistical comparisons of results from DNA adduct analysis of liver samples from oil exposed cod. For details and discussion of results see Skadsheim (2005).





DNA adduct levels measured by ^{32}P-postlabelling in liver samples from Barents Sea cod and North Sea cod exposed to dispersed oil (0.06, 0.25 and 1 ppm) and to 1 ppm oil spiked with 0.2 ppm PAHs and 0.2 ppm alkylated phenols (1+s). The cods were exposed to oil for 1 month, followed by a 2 weeks depuration period. Data from the last day of exposure and the third day of recovery are combined, and pair wise comparison of the different treatment groups was done using the non-parametric Wilcoxon test. Different letters indicate significant differences between groups.